MAR 0 4 2003
PATENT & TRADEMARK OFFICE

Patent Application
Docket No. USF-T127
Serial No. 09/593,629

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Q. Janice Li
Art Unit	:	1632
Applicants	:	Don F. Cameron, Paul R. Sanberg, Samuel Saporta, Joelle J. Hushen, Cesario V. Borlongan
Serial No.	:	09/593.629
Filed	:	June 13, 2000
For	:	Sertoli Cells as Biochambers

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DON F. CAMERON UNDER 37 C.F.R. § 1.132

DR. DON F. CAMERON hereby declares:

THAT, I am the above-named applicant and an inventor of the technology described and claimed in the above-identified U.S. patent application;

THAT, I am a professor of Anatomy at the University of South Florida College of Medicine and a copy of my current *curriculum vitae* is attached hereto as Exhibit A.

THAT, through my years of research, I have kept up to date on the technical literature and maintained contact with experts in the field by participating in professional meetings and seminars, and by direct personal contact. As a result, I am familiar with the general level of skill of those working in the fields of anatomy and physiology, and in particular the physiology of Sertoli cells.

THAT, I have read and understood the Office Actions dated April 24, 2002 and October 23, 2002 in the above-identified application, and the references cited in those Office Actions; and

Being thus duly qualified, do further declare:

Our invention is based upon the discovery that when Sertoli cells and non-Sertoli cells are co-cultured in the presence of a basement membrane preparation such as Matrigel for a period of time, the Sertoli cells are induced to organize, polarizing and epithelializing, such that epithelial-like junctions are formed between adjacent Sertoli cells and the Sertoli cells form a discreet outer wall that defines a lumen, which encapsulates and contains the non-Sertoli cells, as described at pages 12, lines 15-30, page 13, and page 14, lines 1-14, of the patent application. Optionally, the Sertoli cells and non-Sertoli cells can be cultured under microgravity conditions, as described at pages 13 and 14 of the patent application. Advantageously, the resulting cellular arrangement resembles and

functionally mimics the immunoprivileged site of the seminiferous tubule *in vivo*, wherein the Sertoli cells provide a barrier that separates germ cells from system circulation. This structure is known in the art as the "blood-testis barrier". As discussed by Clermont in the Introduction, page XXIV, first column, lines 15-34, and by Byers *et al.*, Chapter 18, pages 432-433, of the Russel and Griswold (1993) publication, The Sertoli Cell (submitted herewith as Exhibits B and C, respectively), adjacent Sertoli cells form a barrier between the basal compartment and the adluminal compartment of the seminiferous epithelium, and the germ cells within the adluminal compartment actually depend largely upon Sertoli cells for support in the form of nutrients, growth factors, and waste disposal. In addition, it has long been known that Sertoli cells provide immunoprotection to adjacent germ cells and other cells, as described in Whitmore *et al.*, *The Journal of Urology*, 134:782-786, 1985; Wyatt *et al.*, *Journal of Reproductive Immunology*, 14:27-60, 1988; Cameron *et al.*, *Transplantation*, 4:649-653, 1990; Selawry *et al.*, *Transplantation*, 5:846-850, 1991; De Cesaris *et al.*, *Biochemical and Biophysical Research Communications*, 186(3)1639-1646, 1992 (submitted herewith as Exhibits D-H, respectively). The ability of Sertoli cells to provide an immunoprotective benefit to co-transplanted cells, facilitating their survival, has also been established, as described in Selawry and Cameron, *Cell Transplantation*, 2:123-129, 1993 (submitted herewith as Exhibit I).

The Office Action raises certain issues with respect to the structure of the biochambers of the claimed invention and the figures in the patent application. Submitted herewith as Exhibits J and K, respectively, are the Cameron *et al.* publications (Cameron *et al.*, *In Vitro Cell. Dev. Biol.–Animal*, 37:490-498, 2001; and Cameron *et al.*, *Ann. N.Y. Acad. Sci.*, 944:420-428, 2001). The Cameron *et al.* (2001) publications describe the co-culture of Sertoli cells and islets, or Sertoli cells and neurons, under simulated microgravity conditions, either in the presence or absence of Matrigel. As described in the abstract and page 491, column 2, the Results section of the Cameron *et al.* (2001) publication (Exhibit J), although three-dimensional aggregates of various sizes formed in both the presence and absence of Matrigel, "the addition of 1% Matrigel induced the reorganization of aggregates (SICAs formed in the presence of Matrigel [SICAmgs]) showing the peripherialization and epithelialization of Sertoli cells and the centralization of islets in association with lumen-like spaces" (emphasis added).

The Office Action also indicates that only the diagrams, and not the photographs of the patent application, illustrate a monolayer. The Office Action states that the patent application does not teach how to control the culture conditions so that only a monolayer of Sertoli cells is formed.

Suitable culture conditions are described in the Examples of the patent application. As indicated above, the biochambers of the invention mimic the general structure of the seminiferous epithelium *in vivo*, and it is expected that this would include the Sertoli cell monolayer structure of the blood-testis barrier. In addition, as described by Djakiew and Onada in Chapter 6, pages 184-187, of the Russel and Griswold (1993) publication (submitted herewith as Exhibit M), Sertoli cells can form confluent epithelial sheets when cultured in the presence of a basement membrane preparation such as Matrigel. Furthermore, as described at page 424 of the Cameron *et al.* (2001) publication (Exhibit K),

> [o]nly in the presence of Matrigel did Sertoli cells segregate to the periphery of the aggregate, leaving the islet cells more centrally located and closely associated with lumen-like spaces. In the latter, Sertoli cells were highly polarized and gave the appearance of a <u>simple</u> <u>columnar</u> <u>epithelium</u>, similar to their morphological and histological appearance *in situ*. This phenomenon of Sertoli cell epithelialization *in vitro* has been observed in conventional culture when Sertoli cells are plated on a Martigel [sic] substrate,[19, 20] but this is the first time that Sertoli cells have been reported to undergo such a dramatic cytoskeletal reorganization when not in direct contact with the substratum. (emphasis added)

The Office Action suggests that the Sertoli cells that form the outer wall of the biochambers of the claimed invention would be a significant barrier for oxygen diffusion to the non-Sertoli cells contained within the lumen. However, quite to the contrary, as indicated in the preceding paragraph, and as described at page 14, lines 1-14, of the application, the cellular arrangement within the biochambers of the invention resemble the blood-testis barrier of the testis. More importantly, the Sertoli cells within the biochambers of the invention exhibit the same physiological properties these cells have in the testis. In addition to immunoprotection, it has long been known that there is direct diffusion of oxygen across Sertoli cells to the luminal compartments within the seminiferous tubule. As observed in the paragraph bridging pages 486 and 487 of Free *et al.*, *Biology of Reproduction*, 14:481-488, 1976 (submitted herewith as Exhibit L), "[t]he lumen of the seminiferous tubules is <u>remote</u> from the <u>blood</u> <u>supply</u> yet does not appear to have a substantially lower oxygen tension than the capillary-rich interstitial tissues" (emphasis added). Therefore, in view of the teachings of the application and the known properties exhibited by Sertoli cells, one of ordinary skill in the art would conclude that the non-Sertoli cells contained within the lumen of the biochambers of the invention would receive oxygen and immunoprotective benefits from the Sertoli cells when co-transplanted as

a biochamber of the invention, thereby facilitating successful cell transplantation and enhancing existing transplantation protocols.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: Don F. Cameron
Don F. Cameron, Ph.D.

Date: 2/24/03

Attachments: Exhibit A: Current *curriculum vitae*
Exhibit B: Clermont, Y., Introduction, page XXIV, first column, lines 15-34 of the Russel and Griswold (1993) publication, The Sertoli Cell
Exhibit C: Byers *et al.*, Chapter 18, pages 432-433, of the Russel and Griswold (1993) publication, The Sertoli Cell
Exhibit D: Whitmore *et al.*, *The Journal of Urology*, 134:782-786, 1985
Exhibit E: Wyatt *et al.*, *Journal of Reproductive Immunology*, 14:27-60, 1988
Exhibit F: Cameron *et al.*, *Transplantation*, 4:649-653, 1990
Exhibit G: Selawry *et al.*, *Transplantation*, 5:846-850, 1991
Exhibit H: De Cesaris *et al.*, *Biochemical and Biophysical Research Communications*, 186(3)1639-1646, 1992
Exhibit I: Selawry and Cameron, *Cell Transplantation*, 2:123-129, 1993
Exhibit J: Abstract and page 491, column 2, the Results section of the Cameron *et al.* (2001) publication
Exhibit K: Page 424 of the Cameron *et al.* (2001) publication
Exhibit L: Free *et al.*, *Biology of Reproduction*, 14:481-488, 1976
Exhibit M: Djakiew and Onada in Chapter 6, pages 184-187, of the Russel and Griswold (1993) publication, The Sertoli Cell

EXHIBIT A

CURRICULUM VITAE

NAME: Don Frank Cameron
BIRTHDATE: August 13, 1947
BIRTHPLACE: El Paso, Texas
MARITAL STATUS: Married - Betsy, 1969
(Katherine, born 1975 and Sara, born 1977)

PRESENT POSITION:

Professor
Department of Anatomy
University of South Florida College of Medicine Tampa, FL 33612
TEL: (813) 974-9434 or 9431
FAX: (813) 974-2058
E-Mail: dcameron@HSC.usf.edu

Professor (Joint Appointment)
Department of Neurosurgery, University of South
Florida College of Medicine, Tampa, FL 33612

Professor (Joint Appointment)
Neuroscience Program
University of South Florida College of Medicine
Tampa, FL 33612

EDUCATIONAL BACKGROUND:

Graduate

Ph.D., Anatomy, 1977
Medical University of South Carolina
Charleston, South Carolina

Dissertation Title:
Structural and Histochemical Response of Adult Sertoli Cells to Peritubular Fibroblasts *in vitro*.
Major Advisor: R. R. Markwald, Ph.D.

M.S., Anatomy, 1972
Medical University of South Carolina
Charleston, South Carolina

Thesis Title:
Histochemical and Ultrastructural Observations on Normal and FSH-Injected Prepuberal Rat Sertoli Cells.
Major Advisor: W. Curtis Worthington, Jr., M.D.

Undergraduate
B.A., Biology, 1969
University of the South (Sewanee)
Sewanee, Tennessee

ACADEMIC POSITIONS:

1999 – present	Professor, Department of Anatomy, University of South Florida College of Medicine (USFCOM), Tampa, FL
1999 – present	Professor, Department Neurosurgery, (Joint Appointment), University of South Florida College of Medicine (USFCOM), Tampa, FL
1999 – present	Professor, Neuroscience Program, (Joint Appointment), University of South Florida College of Medicine (USFCOM), Tampa, FL
1998 - present	Graduate Faculty, Department of Biology, USFCOM, Tampa, FL
1987 - present	Graduate Research Faculty, College of Medicine, USFCOM, Tampa, FL
1997 - 1999	Associate Professor, Neuroscience Program, USFCOM, Tampa, FL
1993 - 1999	Associate Professor of Surgery (Joint Appointment, Division of Urology), USFCOM, Tampa, Florida
1987 - 1999	Associate Professor of Anatomy, USFCOM, Tampa, FL
1986 - 1987	Assistant Professor of Anatomy, USFCOM, Tampa ,FL
1984 - 1986	Doctoral Research Faculty, University of Florida College of Medicine, Gainesville, Fl

1980 - 1986	Assistant Professor of Anatomy and Cell Biology, College of Medicine, University of Florida, Gainesville, FL
1976 - 1980	Instructor in Anatomy, Department of Anatomy and Cell Biology, College of Medicine, University of Florida, Gainesville, FL
1975 - 1976	Teaching Assistant, Gross Anatomy, Histology, Embryology, College of Medicine, Texas Tech University, Lubbock, TX
1974 - 1975	Lab Assistant and Lecturer, Introduction of Microscopic Structural Organization and Gross Anatomy, Medical University of South Carolina, Charleston, SC

TEACHING EXPERIENCE:

1999 - present	Gross Anatomy, School of Physical Therapy, University of South Florida Health Science Center (USFHSC)
1990 - present	Gross Anatomy, University of South Florida College of Medicine (USFCOM)
1990 – present	Graduate Research Topics, USFCOM
1997 - 1997	Histology, USFCOM
1993 - 1995	Anatomical Correlates of Emergency Pre-Hospital Care, Department Anatomy/Hillsborough County Emergency Medical Services, Tampa, FL
1986 - 1990	Gross Anatomy, Course Director, USFCOM
1983 - 1986	Human Systems Development (Human Embryology), Course Director, College of Medicine, University of Florida
1983 - 1985	Gross Anatomy, Laboratory Director, College of Medicine, University of Florida
1981 - 1986	Stomatognathic System (Advanced Head and Neck Anatomy), College of Dentistry, University of Florida

1980 - 1986	Gross Anatomy, Physicians Assistant Program, College of Allied Health Professions, University of Florida
1980 - 1983	Human Embryology, Course Director, College of Medicine, University of Florida
1977 - 1980	Human Organ Development, Director, College of Medicine, University of Florida
1976 - 1986	Gross Anatomy, College of Medicine, University of Florida
1975 - 1976	Gross Anatomy, Histology and Embryology, Teaching Assistant, Texas Tech University School of Medicine

GRADUATE STUDENTS:

Richard Heller, Ph.D., Department of Immunology and Microbiology, University of South Florida College of Medicine (USFCOM), 1987-1991

Jan Dugan, Ph.D., Department of Biochemistry and Molecular Biology, University Florida College of Medicine (JHMHSC), 1991-1994

Francesca Griffin, Ph.D., Department of Anatomy, USFCOM, 1995-1998

Margaret Harvey, Ph.D., Department of Biology, USF, 1995-2000

Joe Johnson, M.S., Department of Biology, USF, 1998-2000

Todd Samelman, Ph.D., Department of Anatomy, USFCOM, 1998-2000

Rebecca Anderson, Ph.D., Department of Bioengineering, University of Florida College of Engineering, 2000-present

Katja Wolski, Ph.D. Department of Anatomy, USFCOM, 2000-present

Lixian Jiang, Ph.D. Neuroscience Program, Department of Anatomy, USFCOM, 2002-present

SERVICE:

Institutional:
Member, Medical Student Selection Committee, USFCOM, 2002-present

Member, Faculty Council Steering Committee, USFCOM, 1999-2003
Chairman, Faculty Search Committee, Department of Anatomy, USFCOM, 2001-2002
President, USFCOM Faculty, 2001-2002
Vice President, USFCOM Faculty, 2000-2001
Chairman, Faculty Development and Evaluation Committee, Department of Anatomy, USFCOM, 2000-2001
Member, Committee on Committees, USFCOM, 1998
Judge, Health Science Center Research Day, USFHSC, 1998-present
Member, Committee on Research, USFCOM, 1997-1999
Member, LCME Self-Study Committee - Governance/Administration USFCOM, 1997-1999
Chairman, Faculty Evaluation Committee, Department of Anatomy, USFCOM, 1996-1997
Member and Secretary, Curriculum Committee, USFCOM, 1994-1997
Member, Oversight Committee of the Anatomy Educational Services, USFCOM, 1991-1994
Member, Oversight Committee, Tissue Culture Room, Department of Anatomy, USFCOM, 1991-1999
Director of Laboratories, Anatomy Educational Services, USFCOM, 1987-1991
Member, Student Affairs Committee, USFCOM, 1990-1994
Member, College Committee to Draft College of Medicine Bylaws, USFCOM, 1990-1991
Chairman, Faculty Association Committee to Draft College of Medicine Bylaws, USFCOM, 1989-1991
Member of the Board and USFCOM Representative, State Anatomical Board of Florida, 1987-1991
Member, Rank and Tenure Committee, Department of Anatomy, USFCOM, 1997, 1989, 1992 and 1993
Member, Faculty Evaluation Committee, Department of Anatomy, USFCOM, 1994-1996
Member, Faculty Search Committee, Department of Anatomy, USFCOM, 1993
Chairman, Faculty Search Committee, Department of Anatomy, USFCOM, 1988
Pre-clinical Medical Student Advisor, Classes 1994 and 1995, USFCOM, 1991-1992
Faculty Advisor, College Republicans, USF, 1994-1996
Member, Search Committee, Juan C. Bolivar Endowed Chair, USFCOM, 1990-1991
Member, Committee for Institutional Grant Administration, USFCOM, 1987-1990
Dean's Task Force, USFCOM, 1989-1990
Chairman, Committee to Draft Faculty Bylaws, USFCOM, 1986-1988
Chairman, Committee to Draft College of Medicine Bylaws, USFCOM, 1988-1989
Judge, Student Symposium on Research in the Medical Sciences, USFCOM, 1989
Board of Directors, Faculty Association, USFCOM, 1987-1989
Member, Faculty Council, University of Florida College of Medicine, J. Hillis Miller Health Sciences Center (JHMHSC), 1985-1986
Member, University Senate, University of Florida (UF), 1985-1986
Faculty Newsletter Committee, JHMHSC, 1983-1986
Preclinical Medical Student Advisor, JHMHSC, 1983-1986
Curriculum Review and Development Subcommittee for Human Systems Development, JHMHSC, 1983-1984

Curriculum Review and Development Subcommittee for Obstetrics and Gynecology, JHMHSC, 1983-1984

Professional:

Consultant, Tekada Pharmaceuticals North America, Inc., 2002-present
Chief Scientific Officer and Director, Saneron CCel Therapeutics, Inc., 2001-present
Co-founder, Saneron Therapeutics Inc., 1999-present
Consultant, American Institute of Biological Sciences, 1999-present
Editorial Board
    Journal of Andrology, 1996-1999
    Frontiers in Bioscience, 1995-present
Editor
    Andrology Newsletter, ASA, 1994-2002
Ad Hoc Editorial Board
    Journal of Histochemistry and Cytochemistry, 1985-1994
    Fertility and Sterility, 1982-1994
Reviewer,
    American Journal Physiology:Endocrinology and Metabolism, 1991-present
    Endocrinology, 1989-present
    Biology of Reproduction, 1989-present
    Journal of Andrology, 1989-present
    Endocrine Journal, 1993-present
    Cell Transplantation, 1993-present
    Anatomical Record, 1998 - present
Ad Hoc Grant Reviewer,
    National Institute of Occupational Safety and Health
    National Science Foundation
    American Diabetes Association
    American Institute of Biological Sciences
    South Plains Foundation
Founding Scientist, Theracell, Inc., 1997
Scientific Consultant, Theracell, Inc., 1997-1998
Consultant and Expert Witness, Department of Professional Regulation, Bureau of Examination Services, State of Florida, 1990-1995
Director, EMS Anatomy, Hillsborough County, Florida, 1987-present
Member, Publications Committee, ASA, 1996-1999
Chairman, Student Affairs Committee, ASA, 1997-2001
Executive Council, American Society of Andrology, 1993-1996 and 1985-1988
Chairman, General Andrology, 11th Annual Meeting of the American Society of Andrology, Grand Rapids, MI, 1986
Chairman, Cell Biology of the Testis, 17th Annual Meeting of the American Society of Andrology, Bethesda, MD, 1992

Chairman, Local Organizing Committee, 18th Annual Meeting American Society of Andrology, Tampa, FL, 1993
Chairman, Local Organizing Committee, XIII North American Testis Workshop (Serono Foundation), Tampa, FL, 1993
Member, Program Committee, 17th Annual Meeting, American Society of Andrology, 1991-1992
Member, Student Affairs Committee, American Society of Andrology, 1986-1990 and 1995-1997
Director, Placement Service, American Society of Andrology, 1986-1990
Member, Diabetes Education Committee, University of Florida, J.H.M.H.S.C., University of Florida, 1984-1986

ACTIVE MEMBERSHIPS:

1997 - present:	American Society for Neural Transplantation and Repair
1985 - present:	Society for the Study of Reproduction
1983 - present:	American Society of Andrology
1982 - present:	American Association of Anatomists
1982 - present:	Sigma Xi

RESEARCH INTERESTS:

General:

- Anatomy, physiology and pathology of the male reproductive system
- Testicular structure and function
- Male infertility
- Sertoli cell-facilitated cell transplantation

Specific:

- Hormonal control of spermiogenesis
- Sertoli cell structure and function
- Sertoli/germ/Leydig cell interactions *in vitro*
- Cell and molecular mechanisms of spermatid binding to Sertoli cells *in vitro*
- Sertoli cell transplantation in diabetes and neurodegenerative diseases

TECHNICAL AND PROCEDURAL ABILITIES:

Light microscopy-bright field, polarization, phase contrast, differential interference contrast, deconvolution
Electron microscopy-scanning and transmission
Tissue morphometry and stereology
Immunocytochemistry-LM and EM
Steroid enzyme histochemistry
Cell and tissue culture

Microgravity cell culture technology.
Gel electrophoresis and Western blot analysis
Velocity and gradient sedimentation cell isolation
Semen, daily sperm production and sperm motion analysis
Print, development and stain technology

PRESENTATIONS AT STATE, NATIONAL AND INTERNATIONAL MEETINGS:

American Association of Anatomists, Dallas, Tx., 1972 (paper presented)
Southern Association of Anatomists, Charleston, S.C., 1972 (paper presented)
Society of Comparative Endocrinology, Beaufort, N.C., 1975
IInd North American Testis Workshop (NIH), Chapel Hill, N.C., 1975 (invited participant)
Third International Congress on Human Prolactin, Athens, Greece, 1981 (paper presented)
American Society of Andrology, Hilton Head Island, S.C., 1982 (paper presented)
American Federation of Clinical Research, New Orleans, LA., 1982 (paper presented)
American Association of Anatomists, Indianapolis, IN., 1982 (paper presented)
American Association of Anatomists, Atlanta, GA., 1983 (paper)
Endocrine Society, San Antonio, TX., 1983 (paper presented)
Third International Congress of Andrology, Boston, MA., 1985 (paper presented)
American Association of Anatomists, Toronto, Canada, 1985 (paper presented)
American Diabetes Association, Baltimore, MD., 1985 (paper presented)
American Society of Cell Biology, Atlanta, GA., 1985 (paper presented)
American Society of Andrology, Grand Rapids, MI., 1986 (paper presented)
American Physiological Society, New Orleans, LA., 1986 (paper presented)
IXth North American Testis Workshop (NIH), Nashville, TN., 1986 (two papers presented)
American Society of Andrology, Denver, CO., 1987 (paper presented)
American Society of Andrology, Houston, TX., 1988 (two papers presented)
Xth North American Testis Workshop (NIH), Baltimore, MD, 1988.
American Society of Andrology, New Orleans, LA., 1989 (paper presented)
Society for the Study of Reproduction, Columbia, OH., 1989 (paper presented)
II International Congress on Pancreatic and Islet
Transplantation, Minneapolis, MN., 1989 (paper presented).
American Society of Andrology, Columbia, SC., 1990 (two papers presented)
American Urological Association, New Orleans, LA., 1990 (paper presented).
XI North American Testis Workshop (NIH), Montreal, Ontario, Canada, 1991 (paper presented)
American Society of Andrology, Montreal, Ontario, Canada, 1991 (two papers presented)
Florida Society for Electron Microscopy, Crystal River, FL, 1992 (paper presented)
American Society of Andrology, Bethesda, MD, 1992 (paper presented)
Society for the Study of Reproduction, Charlotte, NC, 1992 (paper presented)
XIIth North American Testis Workshop (Serono International), Tampa, FL., 1993, (paper presented)
American Society of Andrology, Springfield, IL, 1994 (two papers presented)
American Society of Andrology, Raleigh, NC, 1995 (papers presented)
The Crustacean Society, Ft. Pierce, FL, 1995 (paper presented)

American Society for Neurotransplantation, Clearwater, FL, April 29, 1995 (paper presented)
Society for Neuroscience, San Diego, CA, November 11-16, 1995 (paper presented)
Vth International Congress on Pancreas and Islet Transplantation, Miami Beach, FL, June 18-22, 1995 (paper presented)
American Association of Neurolgical Surgery, Denver, CO, April, 1996, (paper presented)
American Society of Andrology, Minneapolis, MN, 4/27-29/96 (two papers presented)
American Society for Neural Transplantation, St. Petersberg, FL, 4/25-26/96 (two papers presented)
American Society of Andrology, Baltimore, MD, 2/97 (paper)
XIV North American Testis Workshop (Serono), Baltimore, MD, 2/97 (paper presented)
Society for Intrgrative & Comparative Biology, Boston, MA, 1/3-7/98 (paper presented)
PI Workshop, NASA, Houston, TX, 2/26/98 (data presented)
American Society of Andrology, Long Beach, CA, 4/28, 1998 (paper presented)
American Society for Neurotransplantation, Clearwater, FL, April 29, 1998 (2 papers presented).
Experimental Biology 2000, San Diego, CA, 5/13/00 (paper presented)
American Society of Andrology, Boston, MA, 4/7-11/00 (paper presented)
NASA Microgravity Program IGW, Houston, TX, 4/17/00 (paper presented)
American Society of Neural Transplantation and Repair, Clearwater, FL, 4/27/00 (paper presented)
Diabetes Research Institute, Miami, FL, */*/2000
Bioartificial Organs III, Davos, Switzerland, 10/7/2000 (invited plenary lecture)
American Society of Neural Transplantation and Repair, Clearwater, FL, 5/5/01 (paper presented)
ASME Bioeng., Orlando, FL, 6/28/01 (paper presented)
Tottori University School of Medicine, Department of Urology, Yonago, Japan, 4/15/02, (invited presentation)
American Society of Andrology, Seatle, WA, 4/25/02 (paper presented)
American Society of Andrology, Phoenix, AZ, 4/03 (paper presented)
Endocrine Society, Philadelphia, PA, 6/03 (paper presented)
NASA Microgravity Program IGW, Houston, TX, 4/03 (paper presented)
American Society of Neural Transplantation and Repair, Clearwater, FL, 4/03 (paper presented)

FUNDING:

Florida High Tech Corridor External Matching Grant Fund, (Co-PI),
"Tissue engineered living Sertoli Cells – type I collagen bioactive nerve guide for peripheral nerve repair", 5%, 7/02-6/03, $130,000

NASA,(CoPI),
"The creation of transplantable Sertoli-Neuron-Aggregated-Cells (SNACs) for the treatment of Parkinson's disease", (C0-PI), 30%, 12/00-11/04,$1,200,000

NASA,(PI/CoPI),
"The development and evaluation of a three-dimensional Sertoli-Islet-Cell-Aggregate (SICA) for long-term transplantation therapy, 20%, 12/00-11/04, $600,000 (Renewal)

NASA,(PI),
"Development of an Insulin Secreting, Immunoprivileged Cell-Cell Aggregate Utilizing the NASA Rotating Wall Vessel", 30%, $427,000, 6/97-5/01

NIH, SBTTR, (Co-Investigator),
"Sertoli cell co-transplants in Parkinson's disease", 5%, $171,593, 4/1/2001-3/31/2002

Theracell, Inc., (Co-PI), "Sertoli Cell Research", 6/95-5/98, $67,789

National Health and Research Council (Aust), (AI) "Hormonal control of spermatogenesis", 5%, 1998-2003

USFCOM, Teacher Quest, Marketta Gouge, $5,000, Summer, 1997

NIH, RO1 NIDDK, (Collaborator), "Islet transplantation in the Rhesus monkey", $314,000, 6/1/93-5/31/96

USFCOM, Teacher Quest, Joelle Hushen, $5,000, Summer, 1997

USFCOM, Teacher Quest, Joelle Hushen, $5,000, Summer 1996

USFCOM, "Summer research support for medical student, (P. Patel, 2M), $2,000, 6/94-9/94

USFCOM, "Summer research support for medical student", (J.P., 2M), $2,000 6/94-9/94

VAMC Merit 361-36-67-35, (Collaborator), "Intratesticular islet xenografts in the diabetic rat", $500,000, 9/89-8/94

USFCOM, "Summer research support for medical student, (Renda Brownell, 2M), $2,000, 6/93-9/93

USF Research and Creative Scholarship Grant, (Co-PI), "Control of LHRH secretion for a clonal cell line", $7,500, 5/1/91- 4/30/92, $7,500

USF Research and Creative Scholarship Grant, (PI), "Hormonal regulation of spermatid binding to Sertoli cells", $7,500, 6/1/91 - 5/30/92, $7,500

NIH Biomedical Research Support Grant, 2 S07 RR RR05749-18, (PI), "Sertoli junctions in vitro", $6,000, 4/90-3/91

USF Research and Creative Scholarship Grant Program, (PI) -- $7,175 -- 12/1/90-12/1/91

USFCOM Research Equipment Grant, (Co-user), $14,000, 1/90

NIH Biomedical Research Support Grant S07 RR05749, (PI), Sertoli/Spermatid Interactions In Vitro", $7,597, 4/87-3/88

NIH R01 AM 29951-04A1, (Co-PI), "Testicular Dysfunction in Diabetic Man and Rat", $224,965, 9/85 - 8/88

NIH R01 HD 19742-01, (Co-PI), "Effects of hyperprolactinemia on testicular function", $247,322, 12/84-11/87

USF Multi-User Research Equipment Grant, (Co-PI), $11,000, 10/86

NIH R01 HD 11563-01A3, (PI), "Varicocele", $99,427, 4/82-3/85

DSR Institutional Seed Grant, (PI) -- $1,500 -- 3/80-2/81

NIH Biomedical Research Support Grant S07 RR05362, (PI) -- $2,500 4/80-3/81

Bristol Drug Co., (PI) -- $5,455 -- 3/80 - 12/80

American Cancer Society Institutional Grant, (PI) -- $1,000 -- 8/77-6/78

NIH Biomedical Research Support Grant, (PI), -- $3,900 -- 3/77- 2/78

PUBLICATIONS:

Abstracts:

Cameron, D.F., R.R. Markwald and W.C. Worthington, Jr., 1972. Ultrastructural and histochemical effects of FSH on Sertoli cells of prepuberal rats. Anat. Rec. 172:284 (A).

Cameron, D.F. an d R.R. Markwald, 1976. A morphological profile of "in vitro" Sertoli cells and their response to germ cells. Anat. Rec. 184:3, 369 (A).

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EXHIBIT B

Introduction to the Sertoli Cell

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A question that may be asked at the onset is should a book on the Sertoli cell is to be added to the plethora of books recently published in the field of reproductive biology? Obviously, the purpose is to collect under the same cover the maximal amount of information on an interesting cell closely related to differentiating germinal cells in a rapidly developing area of research. In addition to these commonplace reasons, the fact is that the Sertoli cell is unique and fascinating in many respects and excites the interest of biologists in general and of reproductive biologists in particular. On the one hand, the Sertoli cell possesses distinct morphological features that give it a cytological "personality" which is familiar to most investigators working on the testis; if it is not, then the present book will certainly fill this gap! On the other hand, since this cell performs a number of well-integrated, specialized, and even unusual functions, it has a mysterious aura that stimulates curiosity. The purpose of the present introductory chapter is to present a general overview of the structural and functional features of Sertoli cells and, concurrently, before the reader plunges into the details and intricacies of each chapter, introduce the main topics that will be covered in the present book.

Some Structural Characteristics of Sertoli Cell

For the sake of simplicity, the fully differentiated Sertoli cell of an adult mammal, that is the rat, will be used here as a model. Information on the differentiating Sertoli cells will be presented in Chapters 3 and 4 and comparative descriptions of Sertoli cells in Chapters 30, 31, 32, and 33.

Everyone is familiar with the flamboyant appearance of these cells which was well represented by Sertoli himself [1]. The following few main structural features will be mentioned at this point (details are given in Ch. 1, 2, 13, and 32.). The Sertoli cell is a tall (75-100 µm) simultaneously columnar and stellate cell with a base solidly attached to a basement membrane, an apex that reaches the tubular lumen, and numerous lateral and apical veil-like processes extending between and around every germinal cell (Fig. 1). This shape, already well presented by the remarkable description of Sertoli [1] is not obvious in stained sections of testes examined with the light microscope, since the fine lateral and apical processes are masked by the germinal cells. The detail of the Sertoli cell structure was revealed by using the electron microscope,

although here, due to the large size of the cell, only small fragments of the cytoplasm could be seen in a single electron micrograph and the structural characteristics of Sertoli cells had to be reconstituted from numerous photographs and sometimes through laborious reconstructions from serial sections (Ch. 1).

As to the composition of the profuse cytoplasm, most organelles are well represented (Fig. 1). The large polymorphous nucleus with a pale dusty chromatin and a large nucleolus is readily identified toward the lower half of the seminiferous epithelium. The rest of the cytoplasm includes an extensive and continuous system of cisternae of endoplasmic reticulum, a large peri- and supranuclear Golgi forming a continuous network, polymorphous mitochondria, an impressive endosomal-lysosomal apparatus, and an extensive cytoskeleton composed of filaments and microtubules. In the rat a pair of centrioles and peroxisomes have yet to be demonstrated. While there are innumerable small vesicles (of the 50-100 nm range), many serving as carriers of various types of material, there are characteristically no secretory granules. Thus, there is seemingly no storage granules in a cell which is now well recognized as a secretory cell (Ch. 8). Finally, the cytoplasm contains lipidic inclusions and, in the human Sertoli cell, protein crystals. One cytoplasmic feature specific to the Sertoli cell, is a complex formed by flattened cisternae of endoplasmic reticulum and bundles of actin filaments located next to the plasma membrane facing either adjacent Sertoli cells or spermatids (Figs. 1). This system referred to as an "ectoplasmic specialization" will be analyzed in several chapters of this book (Chs. 2, 16). Thus, with the exception of the latter specialization, the composition of the Sertoli cell's cytoplasm is not extraordinary!

The originality of the Sertoli cell lies elsewhere. It lies in the fact that the overall three-dimensional configuration of the cell, as well as the distribution and amount or volume of the various cytoplasmic elements, undergo a marked cyclic evolution which relates to the cycle of the seminiferous epithelium. This bona fide "Sertoli cell cycle" will be extensively analyzed in the present book at both structural and functional levels (Chs. 13, 14, 15, and 32). It should be added that this relatively short Sertoli cell cycle, the exact duration being known for many animal species (it varies from 8 to 16 days, depending on the species) has in numerous mammals a longer, endocrinologically controlled, seasonal cycle (Ch. 15). It will become obvious that the Sertoli cell's short cycle results from the association of Sertoli cells with the 4 or 5 generations of germinal cells forming the seminiferous epithelium. Therefore, the Sertoli cell's cyclical modifications cannot be dissociated from the cyclical events taking place in the whole seminiferous epithelium. Indeed, on the one hand an "autonomous" Sertoli cell cycle taking place in absence of germinal cells either *in vivo* or *in vitro* has yet to be demonstrated, while on the other hand, there are some data indicating that in absence of germinal cells, there is no Sertoli cell cycle.

The corollary of the existence of an *in vivo* Sertoli cell cycle is that to be fully understood, this somatic cell has to be viewed in its relation to germinal cells. However, this does not exclude the interest of *in vitro* studies on isolated Sertoli cells to clarify certain metabolic or other basic biological processes (e.g., secretion, regulatory mechanisms of gene expression, etc.) taking place therein. Indeed, the mass of data recently collected on Sertoli cells resulted from such *in vitro* studies (Chs. 5, 6, 8, and 9). Nevertheless, to be fully meaningful, these data will have to be correlated sooner or later to the *in situ* conditions in which the germinal cells and possibly other elements (e.g., myoid cells, Ch. 21) modulate the biological activity of Sertoli cells.

Major Functions of the Sertoli Cell

The main interest of Sertoli cells lies in their multiple specialized functions, many of which are carried on simultaneously. For the time being, seven main functions can be underscored, most of which are related to the differentiating germinal cells (Fig. 1). Obviously, additional functions will eventually be disclosed as a result of cell and molecular biological investigations on the seminiferous tubules. Let us review briefly these activities and some of the biological queries that they raise.

The first and most obvious function of these cells, recognized by Sertoli himself, is the *supportive or sustentacular* role in maintaining in place the clones of differentiating germinal cells located in the adluminal compartment of the seminiferous epithelium, that is, the spermatocytes and spermatids. Apparently accepted as evident, this function has yet to be explained at the molecular level. Indeed, this attachment process requires the formation and dissolution of junctional devices between Sertoli cells and spermatocytes, about which relatively little is known (Ch. 16). The same may be said about the potential existence and exact role of binding glycoproteins at the interfaces between Sertoli cells and germinal cells (Ch. 18). Similarly the complex process by which elongating spermatids are pulled into deep recesses or crypts formed in the apical part of the Sertoli cells (Fig. 1) and kept in this location during the greater part of spermiogenesis is still unexplained. There is thus clearly much that is unknown concerning this *sustentacular* role of Sertoli cells.

A second function assigned early to Sertoli cells is its capacity to internalize or phagocytose and eliminate residual cytoplasmic bodies that detach from late spermatids at the time of spermiation [2](Fig. 1). Recent data revealed that this phagocytic activity is only one facet of the *endocytic function* of Sertoli cells. Indeed an active pinocytosis or fluid-phase endocytosis takes place at the apex of Sertoli cells. This fluid-phase endocytosis results in the formation of lysosomes which eventually fuse with the phagosomes and contribute to their elimination from the seminiferous epithelium. Thus, phagocytosis and fluid-

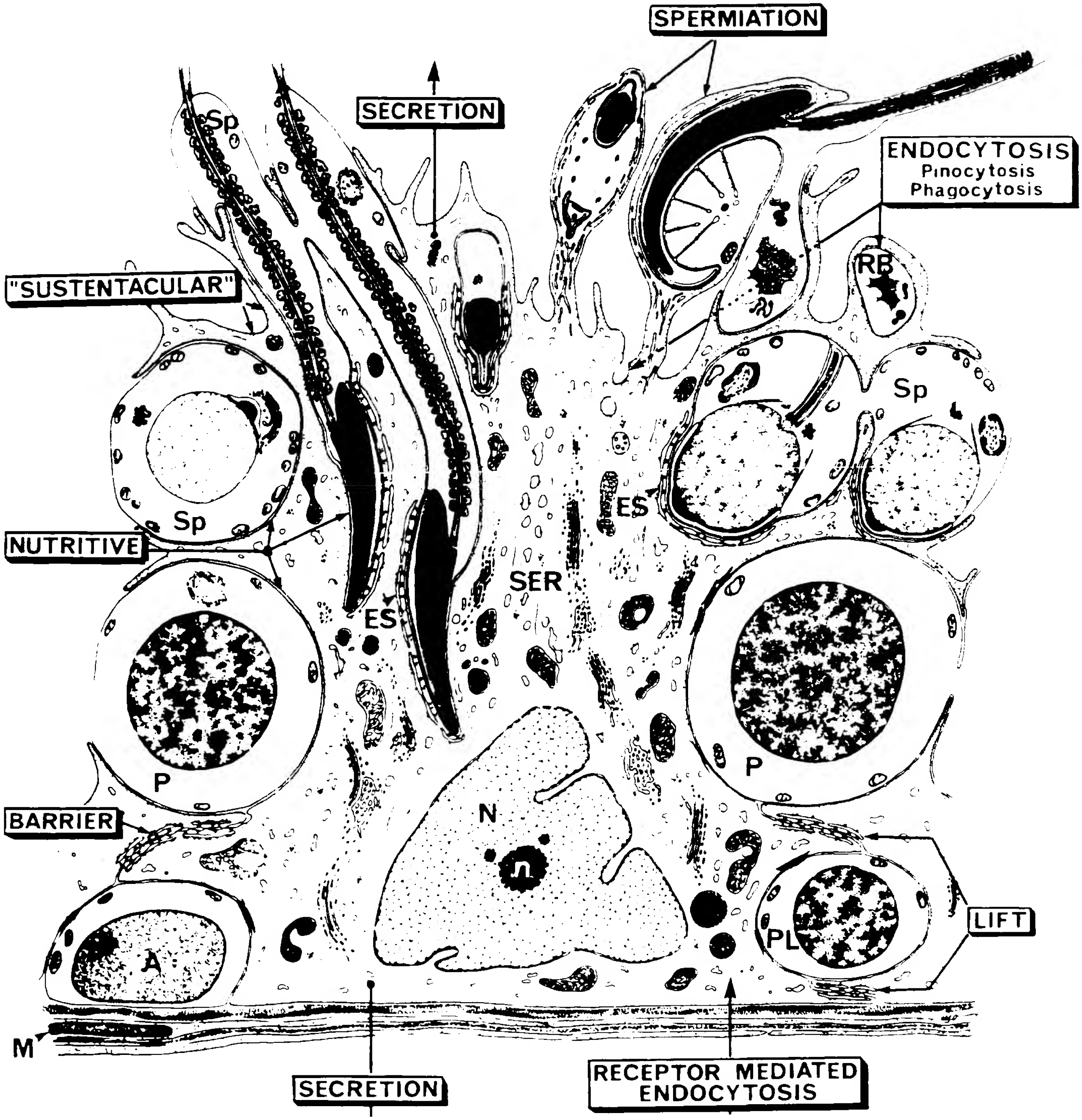

Figure 1. Diagram showing the relationship of a Sertoli cell with the various germinal cells composing the seminiferous epithelium of the rat. The main functions of the Sertoli cell are also indicated. Labels: N, nucleus and n, nucleolus of a Sertoli cell (SER). A, spermatogonium; Pl, preleptotene spermatocyte; P, pachytene spermatocytes; Sp, spermatids; ES, ectoplasmic specialization, RB, residual body; M, myoid cell.

phase endocytosis are well integrated cyclic processes in this cell (Ch. 13). In addition, receptor-mediated endocytosis takes place along the basal plasma membrane (Fig. 1). Many questions may be raised in relation to this endocytic activity. For example: A) What is the exact nature of the recognition mechanism which permits the selective internalization of residual bodies? B) Is there a recycling of the breakdown products resulting from the lysis of residual bodies? C) What are the intracytoplasmic routes and mechanisms of targeting for the lysosomal glycoproteins from the Golgi apparatus to the lysosomes; and finally D) What is the exact nature of the molecular mechanisms controlling this cyclic endocytic-phagocytic function?

A third function of Sertoli cells that has been disclosed by the combined efforts of electron microscopists and physiologists is that adjacent Sertoli cells, by means of intercellular tight junctions, constitute a barrier to macromolecules, the so-called *blood-testis barrier* (Fig. 1)(see Chs. 18 and 25). This results in the formation of two compartments within the seminiferous epithelium, a basal compartment containing spermatogonia and early spermatocytes and an adluminal compartment containing meiotic spermatocytes and spermatids at various steps of spermiogenesis. One consequence of such a barrier is that Sertoli cells must be involved in the transport of substances from the basal compartment to the germinal cells present in the adluminal compartment. With the exception of metallic ions, iron and copper, transported by transferrin and ceruloplasmin respectively, very little information is presently available on the transport role of Sertoli cells. Furthermore, the exact biological significance of a blood-testis barrier in terms of the differentiation of the adluminally located germinal cells still appears unclear.

There is a Sertoli cell function which has a tendency to be neglected and that is its role as a "*lift*" of early meiotic spermatocytes from the basal to the adluminal compartment (Fig. 1). Mechanically, this means that lateral veil-like processes from adjacent Sertoli cells must form and extend under clones of early spermatocytes (i.e., a large group of spermatocytes interconnected by open intercellular bridges) and re-establish contact and attach to each other by new linear tight junctions. Concurrently, the tight junctions above the germinal cells must dissolve to permit the release of spermatocytes into the adluminal compartment. This perfectly timed and synchronized process involving simultaneously a large number of Sertoli cells is in fact highly selective since only spermatocytes are "lifted", while spermatogonia remain attached to the basement membrane. This mechanism obviously requires the recognition by the Sertoli cells of the spermatocytes to be transported and little, if anything, is known about the molecular mechanisms involved in this process.

The active involvement of Sertoli cells in the release of spermatozoa from the seminiferous epithelium or *spermiation* has been well illustrated by several electron microscopic studies (Fig. 1). This phenomenon is complex and includes three distinct phases. During the first phase there is the expulsion of the late spermatids from the Sertoli cell's crypts, and therefore, the migration of the spermatids from the depth of the seminiferous epithelium toward the lumen. During the second phase, the heads of the spermatids individually encapsulated by large Sertoli cell processes are retained for an appreciable period of time along the surface of the seminiferous epithelium. During the third and last phase, the spermatids' heads disengage from the Sertoli cell apical processes but the Sertoli cells selectively retain and phagocytose the cytoplasmic surplus or residual bodies that detach from the spermatozoa. Each one of these three phases about which there are numerous detailed structural descriptions presuppose several complex cell biological and molecular phenomena about which there are discussions and speculations but no complete explanations so far (Ch. 12).

The *nutritive* function of the Sertoli cells, i.e., the delivery of nutritive substances (sugars, amino acids, lipids, metallic elements, etc.) to the germinal cells, is generally considered plausible because of the close association of Sertoli cell processes with every single germinal cell (Fig. 1). Furthermore, the meiotic spermatocytes and spermatids develop in the adluminal compartment as previously mentioned, and are thus dependent on Sertoli cells to have access to nutritive substances. The transfer of vitamin A derivatives and of iron to the germinal cells have been analyzed in some detail (Ch. 10 and 21). Another demonstration of the possible delivery of Sertoli cell products into spermatids was derived from an electron microscopic study in which it was shown that Sertoli cells develop finger-like processes that invaginate the cytoplasm of elongated spermatids and subsequently fragment into pieces that are incorporated in digestive vacuoles within the spermatids. This appears to be a possible delivery pathway of Sertoli cell breakdown products into metabolically active spermatids (Ch. 2). Tritiated labeled sugars or amino acids either injected intravascularly or intratesticularly rapidly reach spermatocytes and spermatids in which they are incorporated into newly synthesized proteins or glycoproteins as demonstrated by radioautography. Whether or not Sertoli cells are involved in the transport of these labeled substances has yet to be fully documented.

The *secretory* function of Sertoli cells has received considerable attention during the past two decades. Several chapters of the present book will present the impressive accumulation of information on this topic (Chs. 7-11). It is now well recognized that Sertoli cells massively secrete a wide variety of proteins as well as water, ions, and many other substances, both at their apical and basal poles (Fig. 1). Thus, evidence for the secretion of proteins such as SGP-1 and SGP-2, androgen binding proteins, metal carrier proteins, proteases and inhibitors, hormones, growth and paracrine factors of various types has rapidly accumulated recently. Most of this novel information was derived from *in vitro* investigations

on isolated and immature Sertoli cells and the exact significance and relative importance of these data in adult animals and *in vivo* remain to be clarified in many instances. It is obviously the value of the present book to collect at this point in time and in a single locus, data and their interpretations on this major aspect of the biology of Sertoli cells.

It should be emphasized at this point that the above mentioned well-integrated functions are targeted mainly to the maintenance of spermatogenesis. This represents an impressive record involving a multiplicity of cell biological and molecular processes that demonstrate the fascination exerted by this cell on biologists. In fact, this "mother-like" behavior of Sertoli cells vis-à-vis all germinal cells is such that it led several investigators in the past to postulate the existence of an ultimate Sertoli cell function which is that this cell *directly* regulates the differentiation of the various generations of germinal cells composing the seminiferous epithelium. In other words, the Sertoli cells would produce a multitude of factors, since a multitude of regulatory mechanisms are necessary to permit the differentiation of spermatogonia into spermatozoa, that would directly provoke, regulate, modulate, or synchronize the gene expression in various germinal cells as they evolve through spermatogenesis. This direct intervention of the Sertoli cell in germ cell differentiation would be responsible for the existence of this remarkable histological process known as the cycle of the seminiferous epithelium. There is as yet no evidence to support this notion, although it appears evident that the various functions of the Sertoli cell *indirectly* permit the unfolding of the genetic program taking place during the differentiation of germinal cells.

Regulation of Sertoli Cell

The regulation of Sertoli cell activities constitutes a rapidly developing although difficult field of research that is well presented in the present book. *Intratubular influences* on the Sertoli cell are evident and multiple. Thus, it is apparent that the germinal cells themselves regulate the Sertoli cell activities, although the exact nature of the regulatory factors and mechanisms are still enigmatic (Chs. 16 and 23). The influence of myoid cells present in the tubular lamina propria on Sertoli cells has been demonstrated *in vitro*, but the exact nature of the influence on the biology of Sertoli cells *in vivo* and in adult animals still needs clarification (Ch. 21). Similarly the influence of the basement membrane on the Sertoli cell polarity has been well documented by *in vitro* studies. However the exact role of the basement membrane on the physiology of the fully differentiated Sertoli cell *in vivo*–once its polarity is established-remains to be clarified. Finally, as in many epithelia, adjacent Sertoli cells present gap or communication junctions at their interfaces. The physiological implications of this intercellular coupling remain to be deciphered (Ch. 19).

Extratubular influences on the Sertoli cells have long been recognized. Indeed, the stimulatory role of the gonadotrophins on Sertoli cells either directly through FSH or indirectly through LH via the androgens secreted by the neighboring Leydig cells (Ch. 23 and 24) has been known for a while. The exact nature of the stimulatory process and the downstream consequences of these stimulations at the molecular level still constitute active and fundamental fields of investigation.

Lastly, since the Sertoli cells are readily accessible to blood-borne substances, including toxins, their cytopathological responses to adverse conditions or substances constitute a field of their own which, to be fully appreciated, has to be related to the normal Sertoli cell structure and cytophysiology (Chs. 27-29).

Conclusions

As the reader progresses through this book and from what has just been said, he or she will not fail to be impressed by the remarkable and unique structural and functional characteristics of the Sertoli cells. Sertoli himself would be amazed at the interest raised by the "cellule ramificate" that he discovered on the tip of his scalpel under his then recently invented compound microscope. This book will present the sum of the information gathered over the years by a rapidly increasing number of investigators who now have access to the most sophisticated analytical methodologies. At the same time, the reader will discover that innumerable questions remain to be answered concerning the biological activities of this somatic cell. Indeed, it is the mysteries that still surround this cell that make it fascinating and interesting to study.

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EXHIBIT C

18 *Sertoli Cell Junctions and the Seminiferous Epithelium Barrier*

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Sertoli cells are found in the seminiferous tubules and form an epithelium that is, in several respects similar to epithelia in other organs. Epithelial cells in general and Sertoli cells in particular can alter the composition of the luminal fluid by pumping fluid into the lumen, by secreting and taking up specific proteins, ions, and other molecules, and by excluding others (reviewed in [1-3]). These vectorial functions are dependent upon the polarized organization of the cells, a characteristic that is mediated by homotypic cell-cell adhesion, the formation of occluding junctions, and by interactions with the underlying basal lamina [4]. The resulting distribution and sorting of the various receptor and transporter molecules to the apical or basolateral domains of the epithelial cell plasma membranes accounts for the vectorial properties of these cells. These are fundamental properties of epithelia in general.

Epithelia in the male reproductive tract can be divided into two groups, those epithelia that are not normally in contact with germ cells and spermatozoa, and those that are. In the first group are the glandular accessory organs, and in the second group are the organs of production and maturation of spermatozoa, the testes and epididymides. The fundamental difference between epithelia in the testis, and epididymis and epithelia in the first group (and all other epithelia) is the presence of meiotic and post-meiotic germ cells in the adluminal and luminal compartment. Consequently, *zonulae occludens* in the testis and epididymis not only serve to permit the vectorial functions described above, but also to segregate germ cells and spermatozoa away from the systemic circulation. Sertoli cell *zonulae occludens* differ from other tight junctions in several important ways. They do not assemble as occluding junctions until puberty, suggesting that the signals responsible for initiating Sertoli cell *zonulae occludens* assembly differ from those in other systems. Sertoli cell *zonulae occludens* must also facilitate and withstand the regular passage of meiotic germ cells from basal to adluminal compartments. Finally, the Sertoli cell tight junctional complex has a unique structure and location within the epithelium. In this chapter the consequences of Sertoli cell *zonulae occludens* assembly will be examined by reviewing the functions of the seminiferous epithelium barrier; then the structure and molecular composition of the Sertoli cell tight junctional complex will be described *in vivo* and *in vitro*. With this as background Sertoli cell tight junctional complex development and dynamics will be examined from a morphological and molecular perspective. Finally, seminiferous epithelium barrier function and Sertoli cell tight junction structure in the damaged testis will be discussed.

Function of the Seminiferous Epithelium Barrier

The existence of a blood-testis barrier was first suggested by the early work of Ribbert [5], Goldman [6], and de Bruyn [7]. In these experiments a number of dyes injected into the bloodstream were not detected in histological sections of the seminiferous epithelium. More contemporary observations of the exclusion of dyes and electron opaque probes from the seminiferous epithelium have demonstrated the presence of a barrier at the base of the seminiferous epithelium consisting largely of Sertoli cell *zonulae occludens* [8-10]. These morphological observations complemented a whole body of physiological studies of whole animals in which a variety of radiolabeled tracers injected into the blood stream were found to be present in much lower amounts in fluid collected from the rete testes [3]. Micropuncture studies of seminiferous tubular fluid have clearly demonstrated the unique composition of this fluid (for review see Ch. 11). These morphological and physiological studies revealed the existence of a seminiferous epithelium barrier and Sertoli cell tight junctions similar to those present in most other transporting epithelia. However, as stated above, unlike other epithelia, the formation of Sertoli cell *zonulae occludens* results in the isolation of a very important population of cells (germ cells) from the systemic circulation. Therefore, the germ cells in the adluminal compartment of the seminiferous epithelium depend largely upon Sertoli cells for support in the form of nutrients, growth factors, and waste disposal. Consequently, one function of the barrier of the seminiferous epithelium is to give Sertoli cells the opportunity to create an environment which is conducive to germ cell development.

Creating a Specialized Environment Essential for Germ Cell Development

The Sertoli cell could create the specialized environment required for successful germ cell development in several ways. It could selectively transport (transcytose) molecules from the systemic circulation across the cell into the adluminal compartment (and vice versa); it could synthesize useful molecules and deliver them directly to the germ cells; it could redirect molecules normally destined for intracellular compartments to the cell surface or to a secretory pathway; and it could selectively exclude factors present in the systemic circulation that may be harmful to germ cell development and perhaps act as an immunological barrier. In addition, it has been stated elsewhere that "rather than (or as well as) regarding the isolation of the testis and the seminiferous epithelium barrier as simply a means of providing certain positive conditions necessary for germ cell development, we should also consider that the cells in the adluminal compartment of the seminiferous tubule actually require the potentially adverse gaseous and nutrient conditions resulting from their isolation to undergo certain stages of meiosis" [2]. The chemical morphogen or gaseous (nutrients) gradients that are likely to be established between the vasculature and lumen of the seminiferous tubule may also be required for germ cell differentiation.

Sertoli Cell Synthesis of Factors Necessary for Germ Cells and Directed Secretion into the Adluminal Compartment All secretory epithelia secrete proteins in a vectorial fashion [4]. Depending upon the organ, proteins

can be secreted predominantly apically or basally. A great deal of attention is being devoted to discovering the molecular structures and events that are responsible for protein sorting in epithelial cells [11, 12]. Although certain aspects of the mechanism whereby integral membrane proteins are sorted to the appropriate plasma membrane domain are beginning to be understood, virtually nothing is known of how constitutively secreted proteins are directed to the correct cell surface. Nevertheless, it is clear from *in vivo* and *in vitro* studies that Sertoli cells secrete certain proteins apically and others basally. For example, *in vivo* androgen binding protein (ABP) is secreted bidirectionally, both into the tubule lumen and into the systemic circulation [13]. The relative proportion of ABP found in the two compartments varies, depending upon the presence of germ cells. Djakiew and Onoda (see Ch. 6) address polarized secretion in *in vitro* models of Sertoli cell culture in some detail. It is clear from these studies that Sertoli cells growing in bicameral chambers also secrete proteins in a polarized fashion. These include, transferrin, ABP, inhibin, sulfated glycoproteins 1 and 2, plasminogen activator and several other proteins detected by two dimensional electrophoresis [14-19]. However, a consensus regarding the direction of secretion of any particular protein has not yet been reached (see Chs. 5 and 6 in this volume for detailed examination of this issue).

Alteration in Intracellular Sorting-Redirection from an Intracellular to Secreted Pathway Several proteins that are secreted by Sertoli cells are not normally considered as secretory proteins. For example, sulfated glycoprotein 1 (SGP-1 [20]) is similar to the 70-kDa precursor to sulfatide/GM1 activator, a molecule which is directed to the lysosomal compartment of other cells, but which is also secreted by Sertoli cells and epididymal cells. Cathepsin L, a lysosomal protease, is secreted cyclically by Sertoli cells and is also secreted by certain tumor cells [21-23]. SGP-1 and cathepsin L may be required for functions within most cells, but in the seminiferous epithelium additional functions may be exerted after secretion by Sertoli cells. It is not known whether these molecules enter the secretory pathway as a result of an intrinsic change in a sorting signal or because of a Sertoli cell specific alteration in the sorting machinery.

Selective Transport and Transcytosis Although it is clear that a variety of ions and other small molecules are transported across the seminiferous epithelium, protein transcytosis by Sertoli cells has not been demonstrated [3]. Many epithelial cells are able to transcytose certain proteins, immunoglobulins and epidermal growth factor, for example, either from the systemic circulation into the lumen, or from the lumen into the systemic circulation [24, 25]. However, as discussed in detail elsewhere [1, 2], it seems that the Sertoli cell synthesizes and secretes many proteins that are found in serum, rather than transcytose them into the adluminal compartment. For example, although transferrin, androgen binding protein and ceruloplasmin are all present in the systemic circulation, the Sertoli cell does not transcytose them into the adluminal compartment, but instead, synthesizes and secretes them. Epidermal growth factor (EGF) is transcytosed by certain epithelial cells [24]. In experiments designed to investigate EGF transcytosis in Sertoli cells it was demonstrated that all bound EGF was internalized and degraded rather than transcytosed [26]. The failure of Sertoli cells to transcytose these molecules may be due either to a lack of the appropriate cellular transcytotic machinery, or to a lack of the appropriate receptor system, or both. For example, Sertoli cells do respond to EGF and the EGF receptor has been demonstrated on the Sertoli cell membrane [26]. On the other hand, receptors for polymeric immunoglobulins are not expressed on Sertoli cells [27]. If Sertoli cells were to have a transcytotic pathway, either from the lumen to the systemic circulation, or vice versa, this pathway would need to have extraordinary fidelity, since any leakage could have disastrous consequences. Because the fidelity of most cellular sorting events is considerably less than 100%, [4, 11, 12], it is most likely that Sertoli cells do not possess the appropriate cellular machinery for transcytosis.

Exclusion of Potentially Harmful Factors from the Systemic Circulation: An Immunological Barrier

In addition to allowing the Sertoli cell to create the appropriate environment for germ cell maturation, Sertoli cell *zonulae occludens* also exclude circulating immunoglobulins and lymphocytes from the adluminal compartment, and prevent antigens from meiotic germ cells from entering the systemic circulation. Although this scenario is at least partly true, autoantigenic germ cells do exist in the basal compartment [28]. Clones of lymphocytes specific for these antigens are also present. In several seasonally breeding animals, meiotic germ cells are present in the seminiferous epithelium at the same time as the *zonulae occludens* cease to exclude permeability tracers [1, 29, 30]. To accomodate these potential compromises other mechanisms must exist to protect the germ cells from the immune system. Anderson and others have demonstrated that immunosuppressive molecules and other immunoregulatory mechanisms are working in the testis to protect the male gamete from the immune system [31]. Interestingly, EGF-like factors and EGF itself are present in the testis and have been demonstrated to be potent immunosuppressants [32, 33]. Clearly, the Sertoli cell *zonulae occludens* is not the only mechanism whereby the immunological barrier of the seminiferous epithelium is maintained.

Structure of the Sertoli Cell Tight Junctional Complex

Several detailed reviews of the fine structure of the various junctional complexes present in the seminiferous epithelium have been published recently [1, 2, 34]. Three chapters in this volume also address different aspects of

junctions. In this section we will briefly discuss the structure of the junctions found in the seminiferous epithelium and compare them with related structures in other organs. In general, junctions in the seminiferous epithelium can be categorized into three types: adhesive, occluding and gap junctions.

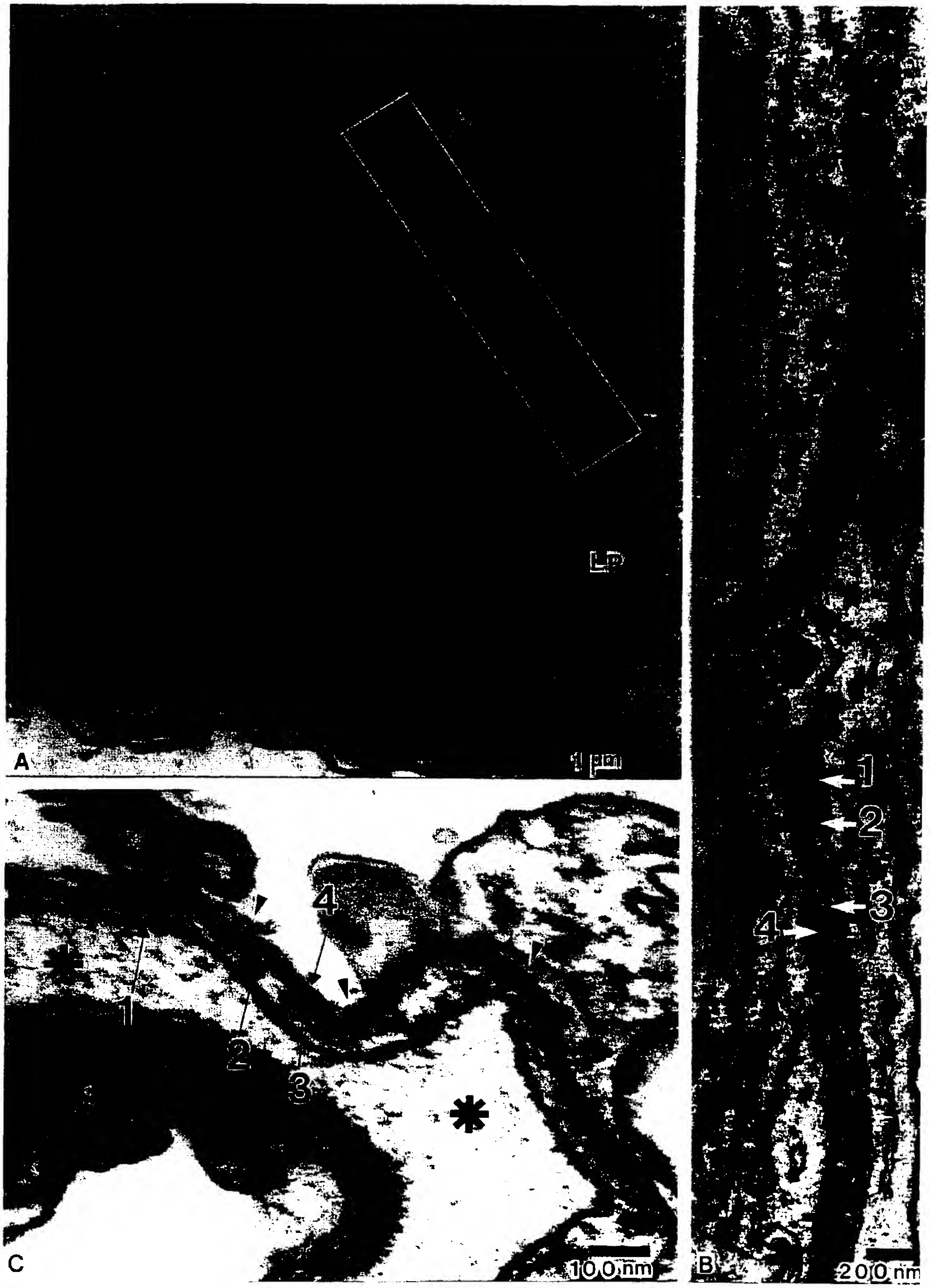
Zonula Adherens and Occludens Junctions

In most mucosal and glandular epithelial cells occluding junctions are present at the apex of the cell. Immediately beneath the *zonula occludens* is a *zonula adherens* (belt desmosome) which is closely associated with a dense circumferential belt of actin filaments. It is now generally assumed that the assembly of the *zonula occludens* is dependent upon the cellular adhesion mediated via the *zonula adherens* [35]. The nature of the molecules involved in this process are discussed elsewhere in this book (see Ch. 20). In contrast to other epithelial cells, the Sertoli cell *zonula occludens* (Fig. 1) is in the basal part of the cell (see [1, 36]) for a discussion of Sertoli cell topography). The basally located Sertoli cell junctional complex has characteristics of both adherens and occluding junctions. Interspersed among points of membrane fusion are densities that probably represent adherens-like junctions. Throughout the junctional region are discrete belts of circumferential actin filaments bordered on the cytoplasmic side by cisternae of endoplasmic reticulum (ER). This plasma membrane/cytoskeleton/ER complex is known as an ectoplasmic specialization (ES) and is unique to the pubertal and postpubertal seminiferous epithelium [37-39](Fig. 2). Vogl, in this volume discusses the ES and its potential as a specialized adhesive junction in more detail (see Ch. 2). The ES is also found associated with round and elongated spermatids, but in these instances, occluding zonules are not present. In the seminiferous epithelium of the testis, it seems that adhesive interactions among Sertoli cells do lead to the formation of tight junctions, whereas ES-mediated adhesion between Sertoli cells and germ cells does not. The lack of occluding zonule formation in this latter situation is probably due to the unilateral distribution of the ES (it is only formed on the Sertoli cell side), and the inability of germ cells to either express or assemble the molecules required for *zonula occludens* formation. This is not surprising since not all epithelial cells form tight junctions even though they all express cell-cell adhesion molecules (CAMs) of one class or another. Transfection of certain epithelial specific CAMs into fibroblasts does lead to homotypic cell adhesion and re-organization of the cytoskeleton, but also does not result in the expression of *zonula occludens*-associated molecules or *zonulae occludens* [40-42]. Nevertheless, elongated spermatids must express adhesive proteins which are able to interact with those CAMs associated with the Sertoli cell ES, because this "junction" is particularly resistant to disruption [37-39]. Whether these molecules are the same as those which are involved in Sertoli-Sertoli cell adhesive junctions remains to be determined (see Ch. 20).

Maculae Adherens (Desmosomes)

In addition to the actin associated *zonulae adherens* other adherent junctions are present in epithelial cells. These *maculae adherens* or spot desmosomes do not completely surround cells but form discrete patches with very strong adhesive properties. Their numbers are directly correlated with tensile strength, and desmosomes are particularly abundant in tissues that are required to be physically robust, such as skin. Desmosomes are associated with keratin intermediate filaments, rather than actin microfilaments. Like other junctions, the assembly of desmosomes is preceded by, and absolutely requires, the expression of primary CAMs. In the testis true desmosomes are not present (see [1] for a discussion). However, structures which have some characteristics of these junctions are present: these will be referred to as desmosome-like junctions. Few desmosome-like junctions are found between Sertoli cells but they are quite abundant between Sertoli cells and most germ cells up to round spermatids (for review see [37]). Only a small number of desmosome-like junctions are found between Sertoli cells and spermatogonia, and between Sertoli cells and elongated spermatids (for review see [37]). This form of adhesion is perhaps not conducive to the processes of spermatogonial mitoses, spermiogenesis, and sperm release. The structure of desmosome-like junctions in the seminiferous epithelium differs in several important ways from those of other organs. Like the ES associated with spermatids, the Sertoli-germ cell desmosome-like junctions are, in certain respects, asymmetrical. For example, although membrane densities are found on both cells, the filament association is far more obvious on the Sertoli cell side [37]. The inter-junction line is also poorly defined in seminiferous epithelium desmosome-like junctions. Certain characteristic desmosomal proteins are also not present in Sertoli cells [43]. As discussed elsewhere [2, 44], Sertoli cells switch from keratin to vimentin expression in the neo-natal testis. Since desmosomes in other tissues are invariably associated with keratin filaments, it is possible that the desmosome-like junctions in the testis have a different composition and function. Nevertheless, these spot adhesive junctions are remarkably

Figure 1 (facing page). (A) Micrograph of Sertoli-Sertoli cell tight junctions and junctional specializations. LP-lipid; SC-Sertoli cell; SPC spermatocyte. (B) The boxed area in A is shown at higher magnification and illustrates the characteristic Sertoli cell tight junction organization. The numbers indicate 1) the membrane of the reticular element closest to the plasma membrane, 2) cytoskeletal material, and 3) & 4) the plasma membranes of adjacent Sertoli cells. (C) Much of this organization is maintained in Sertoli cell plasma membrane preparations. These preparations consisted largely of sheets of plasma membrane and associated junctional specializations. ZO-1 immunoreactivity (gold particles-arrowheads) is commonly, but not always, found associated with the cytoplasmic surface of the membrane at sites of membrane fusion. The asterisks in C indicate the dilated lumen of the reticular element associated with the tight junction. (From [48] with permission of the Publisher.)


LP
A
1 µm
1
2
3
4
B
200 nm
4
1
2
3
C
100 nm

resistant to disruption by hypertonic buffers, indicating strong adhesive properties [37, 45].

Gap Junctions

A chapter of this book is devoted to the structure, molecular composition, and function of these important junctions (see Ch. 19). We will not discuss them further here except to state that their assembly is not possible without the close cell apposition mediated by the adhesive junctions discussed above. That is, the intercellular communication and perhaps synchrony made possible by these intercellular communicating junctions cannot exist in the absence of adhesive interactions.

Other Junctions

Other morphologically distinct surface specializations exist in the seminiferous epithelium. Several workers have described a variety of septate and close junctions with some features that are characteristic of *zonulae adherens* and desmosome-like junctions, and others that are not (discussed in [1, 37]). These structures probably represent other forms of adhesive junctions, or given the dynamic nature of the system, perhaps stages in the assembly or disassembly of *zonulae adherens* and desmosome-like junctions. Hemidesmosomes, which mediate adhesive interactions between epithelial cells and the underlying basal lamina, have been described in this location on Sertoli cells, but not on spermatogonia [37].

Structure of Isolated Sertoli Cell Tight Junctions

Sertoli cells cultured at high density on the extracellular matrix gel Matrigel form tight junctions which have associated with them ectoplasmic specializations [46, 47]. Plasma membranes prepared by sequential dounce homogenization from these cultures consist largely of sheets of membranes rather than vesicles ([48]; Figs. 1C, 2). In some cases sheets of junctional specializations complete with membrane kisses, submembranous material, and cisternae of endoplasmic reticulum are preserved (Fig. 2). In general, the appearance of the isolated membranes and junctional complexes closely resemble these structures in the testis (Fig. 1).

Figure 2. Transmission electron micrograph of plasma membrane preparations from mouse Sertoli cells. Note that the preparations consist largely of sheets of plasma membrane and associated submembranous material. Gold particles (arrows) represent ZO-1 localization. This micrograph shows tight junctions and junctional specializations. Arrowheads mark points of membrane fusion, and asterisks indicate dilated cisternae of endoplasmic reticulum. (Reproduced from [48] with permission of the Publisher.)

Molecular Composition of the Junctional Complex

Zonula Occludens 1 (ZO-1)

Stevenson and co-workers identified the first unique component of the tight junctional complex, ZO-1, using monoclonal antibodies generated against tight junction enriched preparations from mouse liver [49]. ZO-1 is a polypeptide with an apparent molecular weight of 225 kD in humans and rodents, although species differences do exist in the mass of ZO-1 [50]. The physicochemical properties of ZO-1 indicate that it is a tightly associated but peripheral component of the tight junction. Its hydrodynamic properties suggest that ZO-1 is a slightly assymetric monomer. In MDCK cells at least, ZO-1 is phosphorylated on serine residues [51]. cDNAs have been cloned from rat and human, and Southern blotting of restriction digests reveals hybridization to single bands indicating the presence of a single gene [51, 52]. However, northern blots of RNA isolated from various epithelial organs show a major band at 7.5 kb, as well as other minor bands at 8-8.5 kb, raising the possibility of alternative splicing. Interestingly, an additional band at 7 kb is observed in the testis [52].

Immunoelectron microscopy shows that ZO-1 is specifically localized 20 nm from the cytoplasmic surface of junctional membrane contacts [53]. The number of ZO-1 molecules per cell is approximately the same as the number of tight junction particles, implying an association [54].

Cingulin

Monoclonal antibodies were also used by Citi *et al.* to identify a second tight junctional component, cingulin [55, 56]. In this case they were attempting to raise antibodies against brush border myosin. In fact, low angle rotary shadowing shows individual cingulin molecules as highly extended coiled coils reminiscent of the myosin rod [55, 56]. Recent sequence analysis demonstrates that cingulin is indeed a member of the myosin family [57]. On immunoblots cingulin antibodies recognize a 140 and 108 kD species, the smaller molecule perhaps representing a proteolytic degradation product of the larger one. The hydrodynamic properties of cingulin indicate that it is a highly assymetric homodimer. Immunoelectron microscopy shows cingulin to be positioned 60 nm from the junctional contact site and rather diffusely localized along the junctional membrane [55, 56].

Other Molecules

Chapman and Eddy [58], again using monoclonal antibody technology, identified a *zonula occludens* associated molecule designated BG9, with an approximate molecular weight of 192 kD. This molecule has not been characterized further. Other molecules which are clearly present in the region of tight junctions, but which are not specific to them, include actin and associated molecules as well as components of the neighboring *zonula adherens* (see Ch. 2). The molecular composition of the tight junction fibril particle itself is still unknown. It has been suggested that it may be lipidic in nature [59] although solvent and detergent extraction studies indicate that this may not be true [52].

Tight Junction Associated Molecules in the Testis

ZO-1 is present in the testis and is localized to the tight junctional specialization at the base of the epithelium ([48] Fig. 1C). In the mature mouse testis the extent of ZO-1 staining associated with tight junctions varied considerably. In certain instances it seemed to be absent all together (Fig. 3). However, observation of many sections indicated that this variation was likely to be the result of differences in both the orientation of the junctional complex within the epithelium, and in the plane of the section. That is, a glancing section through a junctional complex would give the impression of a greater extent of ZO-1 staining than a perfect cross-section. Similarly, at certain stages of spermatogenesis Sertoli cell tight junctional complexes are more perpendicular to the basement membrane than at other stages. Of particular interest is the observation that at certain stages ZO-1 was clearly localized to the Sertoli cell membrane adjacent to round and elongating spermatids, presumably at the ectoplasmic specialization (Fig. 3A). The Sertoli cell membrane associated with advanced spermatids just prior to sperm release was negative for ZO-1 (Fig. 3C).

ZO-1 was also localized to the cytoplasmic surface of Sertoli cell plasma membrane preparations apparently associated with sub-membranous "fuzzy" material, but not with the adjacent endoplasmic reticulum (Fig. 1C, 2). Other areas of plasma membrane as well as contaminating membrane bound organelles were negative. Several images suggested that ZO-1 was localized to the cytoplasmic surface of the membrane adjacent to the sites of fusion as described previously for liver tight junctions [49], although other gold particles were present throughout the tight junctional region. As the immunolabeling was carried out on unfixed membranes, access of the labeling reagents was limited to regions which were somewhat disrupted. By the same reasoning, it was also possible that access of antibodies to the external surface of the plasma membrane was limited. However, it is clear from other studies that ZO-1 does not span the plasma membrane and is only present on the cytoplasmic surface of the tight junction [49, 50].

Immunolocalization of cingulin in adult mouse and rat testes reveals a distribution similar to that of ZO-1 (Figs. 4 and 5). Positive staining is present at basal tight junctions as well as at sites of spermatid-Sertoli cell contact ectoplasmic specializations). In some sections cingulin staining is present on other regions of the Sertoli cell membrane.

Development of the Sertoli Cell Tight Junctional Complex

General Considerations

Unlike tight junctions in all other mucosal and glandular tissues, which are of necessity functional at birth, the Sertoli cell *zonula occludens* does not commence assembly

until puberty (see Ch. 4). The timing of this event is conserved in all mammals in which it has been studied. This suggests that not only is the timely formation of the Sertoli cell occluding zonule important for spermatogenesis, but also, that its premature formation is likely to be detrimental to reproductive success. How early formation of the Sertoli cell occluding zonule may adversely affect reproductive function is not immediately obvious. The authors favor one of the following explanations. Firstly, gonocytes in the pre- and neo-natal testis are located in the central part of the seminiferous cord. During this period these cells are continually being relocated to the basal part of the cord, adjacent to the basal lamina (see Ch. 4). Consequently, by the time Sertoli cell tight junctions have formed at puberty no gonocytes are present in the center of the cord. It is possible that these "stem" cells would remain trapped above the occluding zonule and may not differentiate successfully if premature tight junction formation were to occur. In other words, premature tight junction formation may limit the number of stem cells available for spermatogenesis, ultimately resulting in testes containing fewer germ cells. Secondly, Sertoli cell mitosis ceases around the time of *zonula occludens* assembly. Although these two events may not be directly related it is possible that premature *zonula occludens* formation could result in the early cessation of Sertoli cell proliferation. Since each Sertoli cell is associated with a fixed number of germ cells, a reduction in Sertoli cell number may also lead to lower numbers of germ cells successfully completing spermatogenesis.

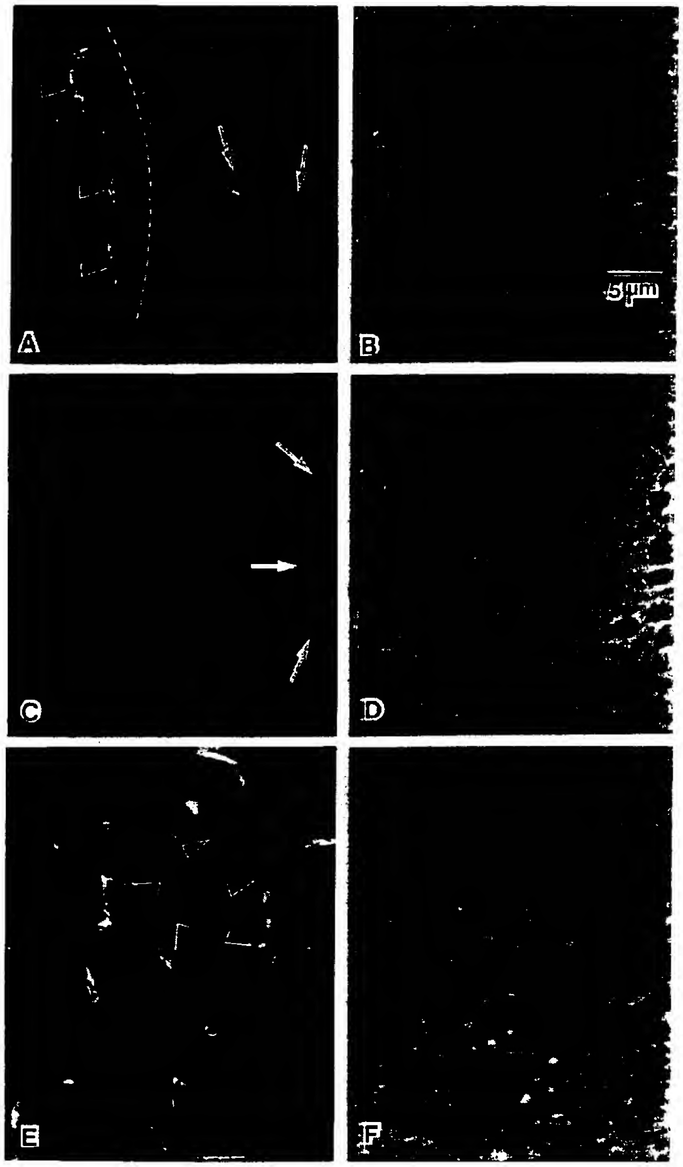


Figure 3. Paired fluorescence (A, C, E) and phase contrast micrographs (B, D, F) of ZO-1 distribution in mature mouse testis. (A, B) stage V of the cycle. Arrowheads indicate tight junctions and arrows point to germ cell. The dashed line marks the basement membrane. (C, D) As spermiation take splace in stages VI-VIII, ZO-1 staining associated with spermatids was greatly diminished. (E, F) In sections cut through the basal part of th epithelium, a polygonal pattern of ZO-1 immunoreactivity was observed, corresponding to the circumferential tight junctions. (From [48] with permission of the Publisher.)

Signals Responsible for the Initiation of Sertoli Cell *Zonula Occludens* Assembly?

The assembly of the Sertoli cell *zonula occludens* coincides with the many other events that result from the pubertal surge in gonadotropic and androgenic hormones. Therefore, it would seem likely that Sertoli cell tight junction formation must be regulated either directly by hormones or indirectly, by the presence of certain germ cells. Several studies have addressed the effects of hormones and germ cells on the formation of the "blood-testis" barrier [60-64]. Surprisingly, neither the absence of germ cells, nor hypophysectomy or treatment with estrogens, prevents or reverses the formation of the "blood-testis" barrier, although its formation is delayed slightly. However, no tight junctions were found in men with hypogonadotrophic hypogonadism and they reappeared after treatment with gonadotropins [64]. FSH, testosterone, phorbol esters, cAMP and temperature were found to regulate the magnitude of the electrical resistance of Sertoli cell monolayers cultured in bicameral chambers [65, 66]. Nevertheless, Sertoli cells isolated from 18 day-old rats do develop tight junctions and an electrical resistance in the absence of hormones. The hormonal manipulations reported in the studies of Janecki *et al.* [65, 66] point to a role in the regulation of tight junction function, but do not, as suggested by these authors, address the nature of the signals responsible for actually initiating Sertoli cell *zonula occludens* formation. Phorbol esters, cAMP, and protein kinase C inhibitors also affect tight junction function in other systems [67]. These sorts of experiments need to be repeated and extended before any definitive conclusion can be reached. Nevertheless, it is possible that events independent of those normally associated with puberty, also need to be taken into account when considering the factors which regulate Sertoli

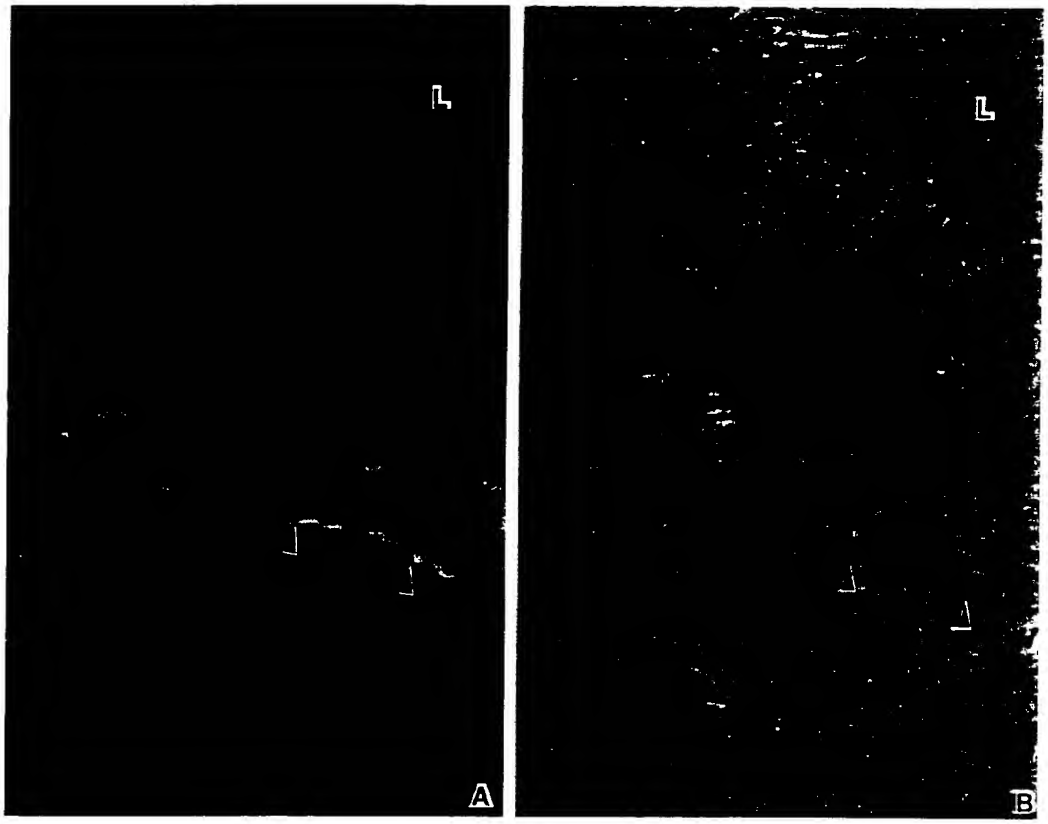


Figure 4. Paired fluorescence (A) and phase contrast micrographs (B) of cingulin distribution in mature mouse testis. Note that in this micrograph staining is largely restricted to the basally localized tight junctional areas (arrowheads).

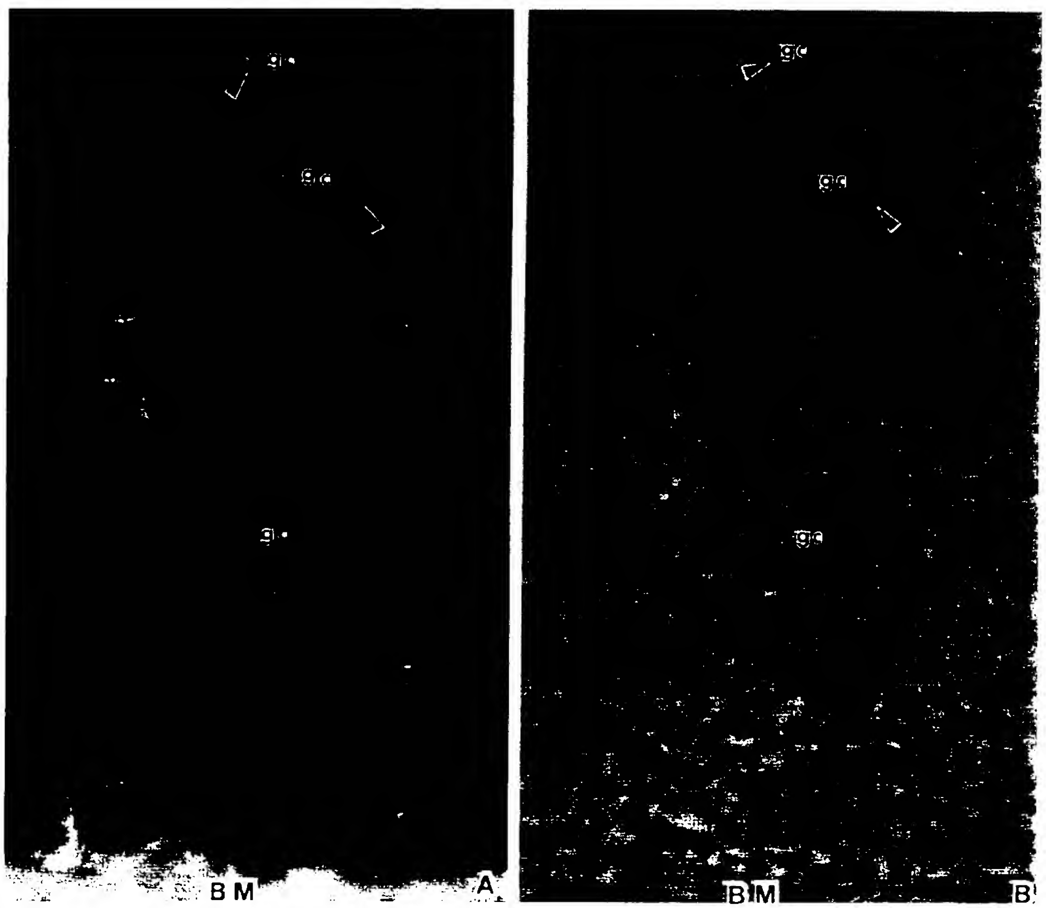


Figure 5. Paired fluorescence (A) and phase contrast micrographs (B) of cingulin distribution in mature mouse testis. In this higher magnification image, cingulin, like ZO-1, is clearly associated at the interface between Sertoli cells, and some but not all germ cells (gc).

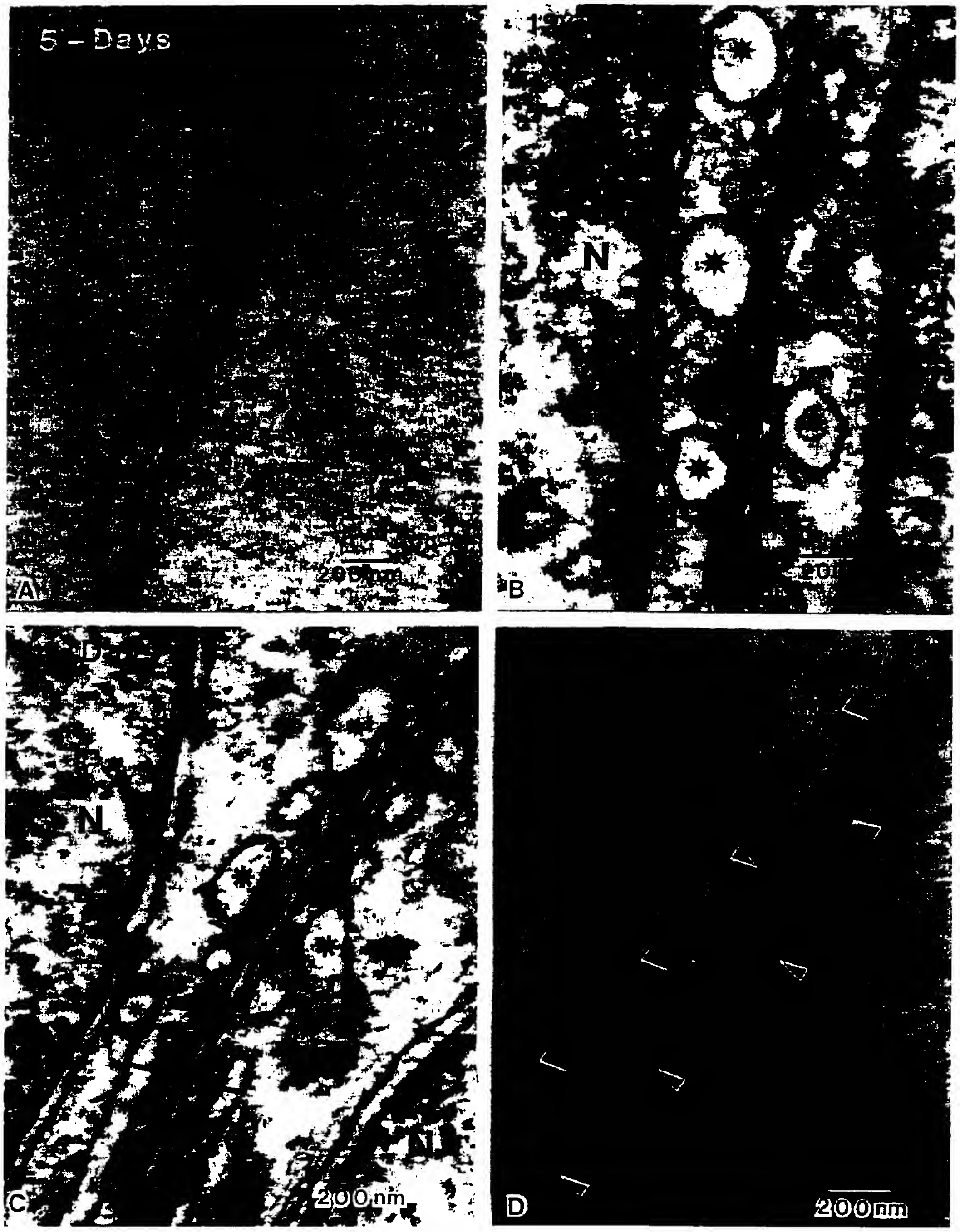


Figure 6. Electron micrographs of developing Sertoli cell tight junctions. N, nucleus; asterisks indicate reticular elements. (A) At 5 days no recognizable tight junctional structures are observed despite the close apposition of adjacent Sertoli cell membranes. In some images it seems that reticular elements are budding from the nearby nuclear membrane to become associated with the developing junction. (B) By 12 days these reticular elements are more organized and fibrous material (cytoskeleton) is recruited to the developing tight junction. (C) By 14 days, tight junctions are almost completely formed, sites of membrane apposition are clearly defined (arrowheads) and the reticular and cytoskeletal elements are quite well organized. (D) By 18-24 days a mature form of tight junction is observed with extensive reticular elements (arrowheads). (From [48] with permission of the Publisher.)

cell tight junction formation. The oft-stated role of the "blood-testis" barrier in isolating the meiotic germ cells from the immune system may give us a clue regarding the nature of this "independent" regulation. It is well established that the mammalian immune system also undergoes an age related maturation, much of our immunity being established early in life. Is it possible that some aspect of the maturation of the immune system, in addition to pubertal maturation, is involved in the regulation of Sertoli cell occluding junction formation?

Sertoli Cell Occluding Zonule Structure During Development.

Sertoli cell focal junctions develop *in utero* in rodents and they are readily identifiable at birth [68-70]. However, only at puberty do these focal junctions assemble into an occluding zonule (see ref [1] and Ch. 4). Freeze-fracture replicas of the newborn mouse testis revealed short linear arrangements of tight junctional particles which appeared to be an indication of the initial formation of the *zonula occludens* [68]. By six days of age focal occluding junctions

were arranged in a meshwork and gap junctions were common. However, an occluding zonule was not present. At later times the meshwork of discontinuous strands formed many parallel occluding zonules which completely surrounded the Sertoli cell [68]. The number of gap junctions decreased as the occluding zonules became arranged into continuous strands. Transmission electron microscopic observations of the five day-old mouse testis (Fig. 6A) showed that occluding tight junctions and associated ectoplasmic specializations were not present on the Sertoli cell plasma membrane. In these animals Sertoli cell nuclei occupy most of the basal regions of the cells and there is a close juxtaposition of nuclear and plasma membranes. Between 7 and 12 days (Fig. 6B) some poorly defined fusion sites can be observed and there is a recruitment of vesicular elements and "fuzzy" material, possibly corresponding to cytoskeleton. By 14 days (Fig. 6C) distinct points of membrane fusion can be observed and filamentous material together with tubules and fusing vesicles of endoplasmic reticulum are situated on the cytoplasmic side of the tight junction. This characteristic organization of cytoskeleton and endoplasmic reticulum associated with the tight junction is known as an "ectoplasmic specialization." Many junctional specializations at this age are fully formed and indistinguishable from the adult. By 24 days many mature tight junctions are found with extensive arrays of actin filaments and reticular elements accompanying the *zonula occludens* proper (Fig. 6D). A similar sequence of events has been described during tight junction formation between Sertoli cells cultured in bicameral culture inserts and after germ cell translocation [1, 29, 71].

Changes in ZO-1 During Development In the absence of recognizable tight junctions (5-10 days) ZO-1 is localized to the apicolateral Sertoli cell plasma membrane (Fig. 7A). As cytoskeletal and reticular elements are recruited to the developing tight junctions, ZO-1 immunoreactivity becomes progressively more restricted to these regions (Figs. 7C-7E).

Movement of Germ Cells Into the Adluminal Compartment: Dynamics of Sertoli Cell Tight Junctions

Perhaps the most studied, but least understood morphological aspect of spermatogenesis is the passage of germ cells from basal to adluminal compartments of the seminiferous epithelium. In the post-pubertal testis this process requires that early spermatocytes are directed past the *zonula occludens* between adjacent Sertoli cells. In pubertal rats pachytene spermatocytes are present in the testes prior to completion of the Sertoli cell *zonula occludens* and assembly of the ectoplasmic specialization. This indicates that the movement of early spermatocytes away from the basal lamina and the subsequent appearance of pachytene spermatocytes in the adluminal compartment in immature rats is not associated with the same occluding zonule dynamics as those same events in older animals. The complex dissolution and reformation events that occur during spermatocyte passage into the adluminal compartment in the post-pubertal animal can then be viewed as a response to spermatocyte migration, rather than its cause. This is not so surprising, but given that the movement of clones of spermatocytes into the adluminal compartment is almost certainly directly facilitated by Sertoli cell adhesive contacts, it points to Sertoli-germ cell desmosome-like adhesive contacts as the important adhesive junction in this process. Another aspect of occluding zonule dynamics that is commonly misunderstood, even though it has been explicitly stated by Russell and others on several occasions, is that spermatocytes may not actually pass through the occluding zonule [37]. Instead, morphological studies show that occluding zonules and associated ectoplasmic specializations move upwards in the plane of the Sertoli cell plasma membrane. Spermatocytes that are associated with the Sertoli cell plasma membrane by desmosome-like contacts also ascend, new occluding zonules assembling as Sertoli cell plasma membranes meet beneath them. Once these are patent, the occluding zonule above the spermatocyte can disassemble. How the same cell can simultaneously assemble and disassemble occluding zonules in different parts of the cell remains a perplexing question, but these events may be coordinated through the interaction of CAMs with the cytoskeleton ([1]; see Ch. 20).

Sertoli Cell Tight Junctions in the Damaged Testis

Testicular damage is most often accompanied by loss of germ cells and many of the characteristic morphological changes encountered following testicular insult are readily observed with the light microscope. At the electron microscope level it became clear that Sertoli cells were also affected and exhibited extensive vacuolization, loss of cytoplasmic ground substance and an increase in the abundance of intermediate filaments (see [34, 37] for review). The appearance of Sertoli cell tight junctions is also altered following experimental cryptorchidism [72]. In this case, the extracellular space within Sertoli cell junctional areas becomes dilated and Sertoli cell ectoplasmic specializations are displaced from the close association with the plasma membrane. As germ cells are lost from the epithelium, the apical Sertoli cell cytoplasm collapses giving rise to a concertina-like appearance of extensively pleated junctional complexes on opposing sides of the plasma membrane (see [34] for a detailed discussion).

In a naturally cryptorchid testis similar concertina-like structures are observed. However, these do not appear to be present on both of the adjacent Sertoli cells, rather the pleated junctions are unilateral. In addition, the concertina-like structures are present predominantly in the apical rather than basal part of the Sertoli cell.

In spite of these quite dramatic changes in junctional morphology, in most cases of testicular damage the barrier

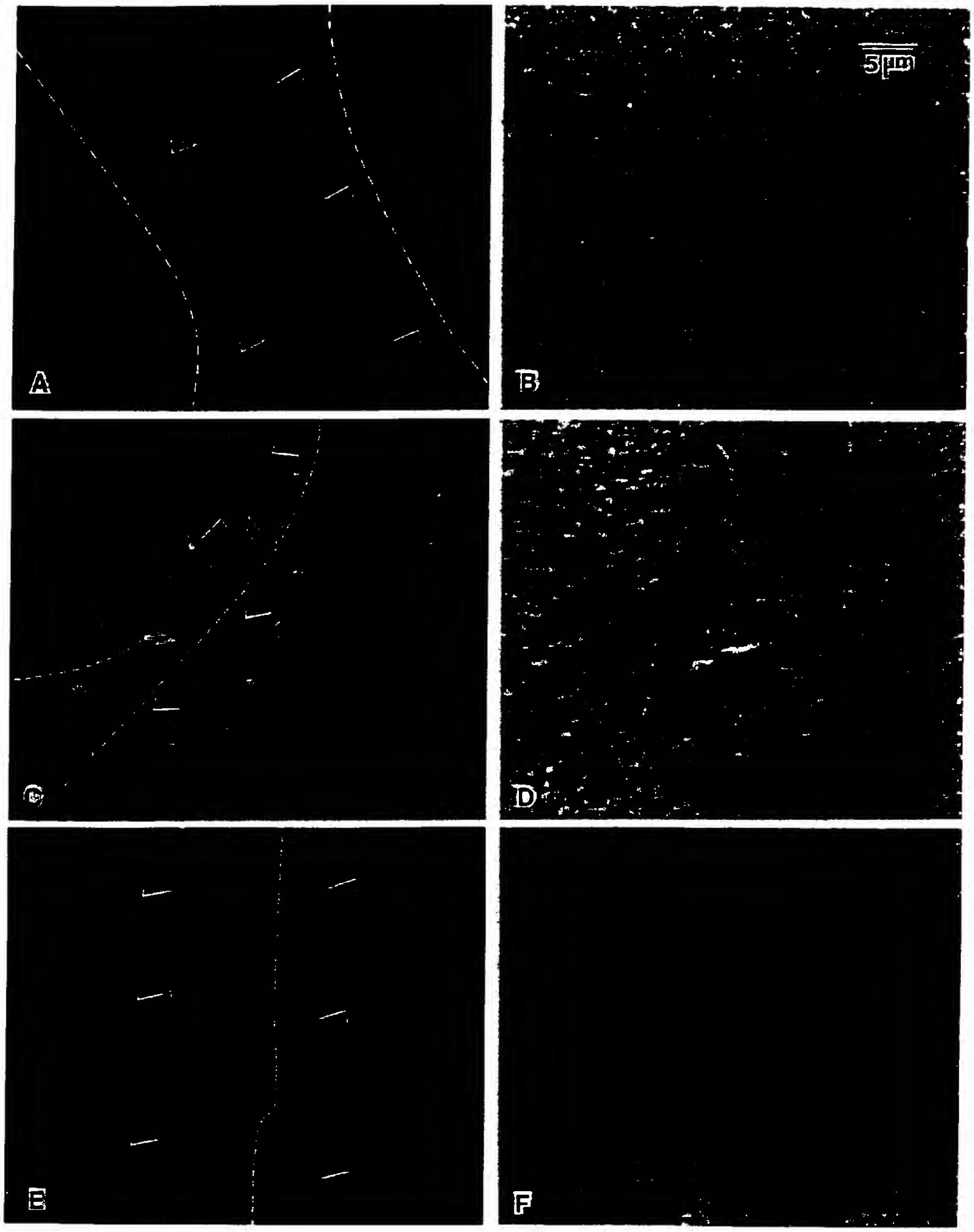


Figure 7. Paired fluorescence (A, C, E) and phase contrast micrographs (B, D, F) of ZO-1 distribution in the prepubertal and pubertal mouse testis. The dashed lines indicate the basement membrane. Note that specific staining (arrowheads) is restricted to the apical aspect of the seminiferous tubule in the 5 day testis (A,B). ZO-1 localization in the 13 day mouse testis becomes more restricted to the basal aspects of the epithelium (arrowheads). At this time tight junctions identified with the electron microscope are forming. Note that staining is also associated with endothelial cells in the interstitium (white arrows). By 18 days (E,F) ZO-1 staining is restricted to the basal aspects of the epithelium. (From [48] with permission of the Publisher.)

functions of the occluding zonule are maintained (see [37] for review). An interesting exception to this is the loss of a permeability barrier following treatment with cadmium, a divalent metal well known for interfering with testicular function [73, 74]. Sertoli cells cultured in bicameral chambers also lose transepithelial resistance after exposure to cadmium [75]. However, low levels of cadmium have recently been shown to specifically interfere with the function of the calcium-dependent cell adhesion molecule E-cadherin in epithelial cells [76]. MDCK cells treated with cadmium rapidly lose the ability to maintain an electrical resistance across the monolayer, an effect that is accompanied by changes in cadherin function and distribution. Therefore, it is possible that the specific effects of low levels of cadmium on Sertoli cell tight junctional permeability are a result of a cadmium induced alteration in the function of a calcium-dependent CAM. However, this is not likely to be E-cadherin since it is not present in the seminiferous epithelium (see Ch. 20).

Other treatments which also result in alterations in Sertoli cell *zonula occludens* structure and/or function include gossypol [77; Figs. 8, 9], cisplatinum [78], and dis-

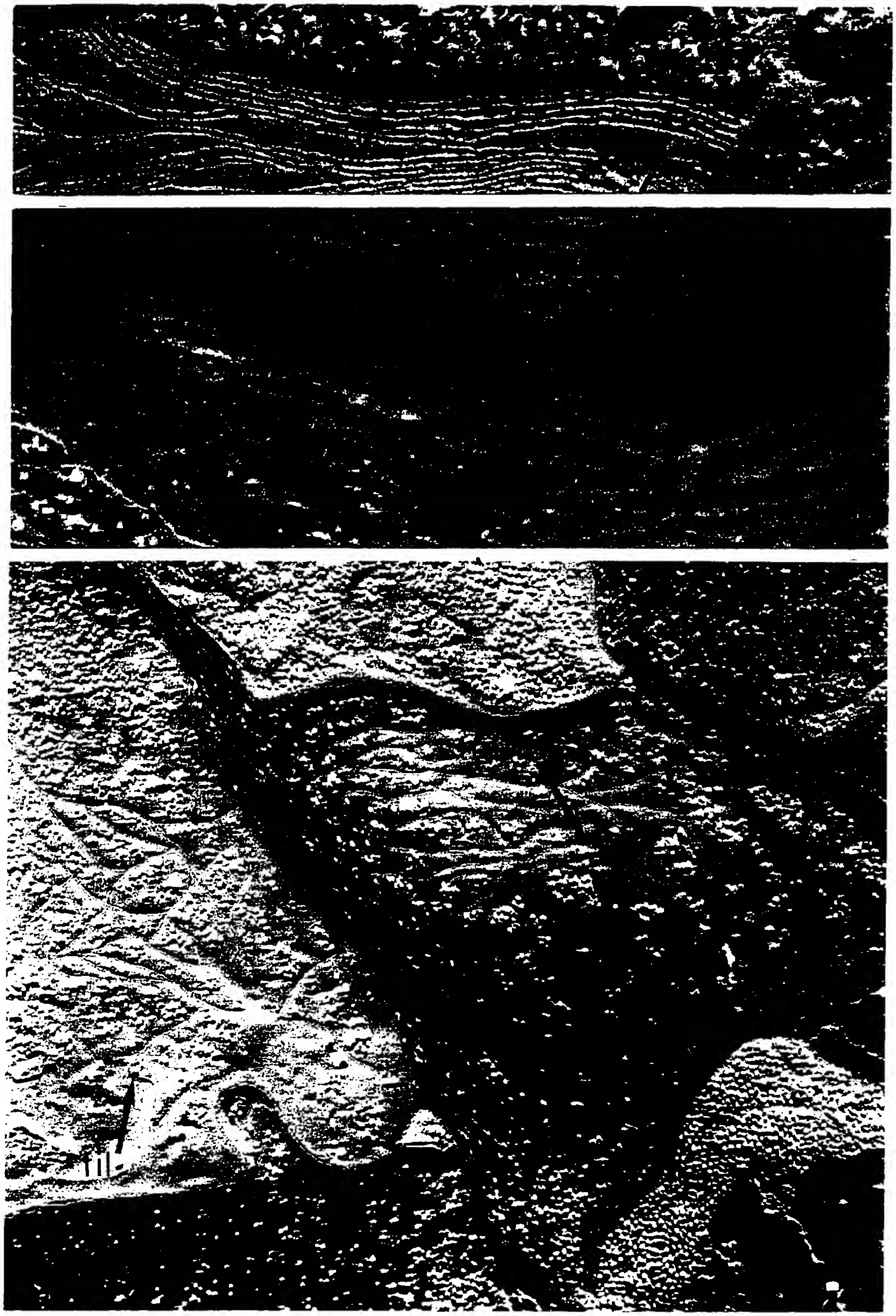


Figure 8. (A) In the normal guinea pig, when the polyene antibiotic filipin (fil) is used as a permeability tracer to test the tight junctions between adjacent Sertoli cells, junctions that consist of continuous strands are not penetrated. (B) Discontinuous zonules composed of incomplete meandering strands restrict to do not completely prevent the passage of filipin (fil). (C) In this freeze fracture replica showing five adjacent Sertoli cell junctional membranes taken from a 31 day-old gossypol treated guinea pig, the junctional strands are discontinuous and permeable to filipin (fil).

ruption of actin filaments with cytochalasin [79]. In the case of gossypol treatment freeze fracture observations (Figs. 8, 9) reveal large distensions between continuous and discontinuous strands of tight junction particles which are no longer impermeable to the antibiotic polyene filipin. In the normal animal only continuous strands are impermeable

Figure 9. Replica of a Sertoli cell junctional area showing large distensions between continuous and discontinuous strands that are permeable to filipin (fil) in a gossypol-treated adult guinea pig.

to filipin (Fig. 8). Freeze-fracture replicas of cytochalasin D injected testes revealed many focal defects in the *zonula occludens* [79]. For example, there was a reduction in the number of strands and a loss of intramembranous particles on both the E- and P-faces of the replica as well as a clustering of particles between the strands. These morphological alterations were reflected in an increase in lanthanum and IgG permeability.

Conclusions

Many structural and functional aspects of the barrier of the seminiferous epithelium have been elucidated. Morphological studies have revealed, in some detail, the ultrastructure of the Sertoli cell tight junctional complex during development, maturation, and during germ cell translocation. Studies, originally *in vivo*, and more recently *in vitro*, have addressed the permeability, polarity, and vectorial secretion properties of the seminiferous epithelium and Sertoli cell monolayers in culture. One central issue has yet to be addressed. The molecular and cellular events accompanying cell tight junction assembly and dynamics of Sertoli cells are almost completely unknown. The recent characterization of two tight junction associated molecules, ZO-1 and cingulin, and their localization to Sertoli cell tight junctions, has provided specific tools with which to begin investigations in this area. Using these tools, and *in vivo* and *in vitro* model systems, useful information can now be obtained regarding several unresolved questions. These question include: 1) What are the signals which initiate assembly of the *zonula occludens* in the prepubertal animal? 2) How are these signals interpreted by the cell in terms of second messengers, alterations in gene expression, and post translation modification (phosphorylation) of tight junction proteins? 3) What is the molecular basis of Sertoli cell *zonula occludens* dynamics during germ cell translocation?

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EXHIBIT D

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THE ROLE OF GERMINAL EPITHELIUM AND SPERMATOGENESIS IN THE PRIVILEGED SURVIVAL OF INTRATESTICULAR GRAFTS

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ABSTRACT

The testis is an immunologically privileged site. Since earlier studies excluded testicular steroid production as an essential factor, the present study evaluates the role of germ cells and spermatogenesis in the privileged survival of allografts within the testis. We used a Sertoli cell-only testis model and adolescent unilateral cryptorchidism in inbred rats to eliminate germ cells and spermatogenesis selectively. Parathyroid allografts were implanted into these sites, normal testes and beneath the renal capsule (a nonprivileged site) in appropriately matched controls. With at least 15 rats in each group, privileged allograft survival was shown to be unaffected by eliminating germ cells and spermatogenesis ($p < .005$). Experimental evidence suggests the presence of an active process which incidentally permits privileged allograft survival within the testis, but which exists teleologically to protect the developing sperm from autoimmune attack. This is in addition to the passive anatomical separation provided by the blood-testis barrier. Our cumulative data strongly implicates the Sertoli cell in this process.

A broad range of in vivo studies by many investigators has demonstrated that the testis is an immunologically privileged site.[1-6] Since germ cells and developing sperm have surface antigens which are recognized as foreign by the immune system,[7, 8] this protective feature of the testis is probably essential to prevent autodestruction of the germinal epithelium and have normal spermatogenesis. We have been investigating the immunological mechanisms of the testis as a privileged site and specifically what feature of the testis is essential to this process. Our most recent hypothesis has been that some byproduct of spermatogenesis or some product of the germinal epithelium creates this privileged milieu. This hypothesis invokes a cell product rather than direct cell-cell interaction because the immunologically privileged region extends outside the blood testis barrier to include the interstitial space (in effect, the entire testis).[9] Since the germinal epithelium and developing sperm are rigorously contained within the seminiferous tubules and yet privileged allograft survival is seen to take place in the interstitial (extratubular) space of the testis, it is logical to propose a diffusable factor produced as a consequence or byproduct of gametogenesis.

To test this hypothesis, there were two arms to our experiment. The role of spermatogenesis was tested by observing allograft survival in longterm cryptorchid testes, and the importance of germ cells was established by observing allograft survival in so-called Sertoli cell-only testes.

MATERIALS AND METHODS

Cryptorchid testes. Lewis inbred male rats (Microbiological Assoc.) were made unilaterally cryptorchid by surgically securing the right testis in an intraabdominal position at 21 days of age. Eight weeks later these rats were parathyroidectomized. Each Lewis rat then received a male Lewis × Brown Norway F_1 (LBNF$_1$) (Microbiological Assoc.) parathyroid gland allograft implanted deep into the cryptorchid testicle or into control sites, using methods described previously.[4] Controls included autografts in all sites and allografts transplanted into the normal testis located in the scrotum or beneath the renal capsule in matched unilaterally cryptorchid animals.

Sertoli cell-only testes. It has been shown that the germ cells in male rat fetuses display increasing radiosensitivity up to 18 days of embryonic life.[10] Between 19 and 21 days they are acutely sensitive, but thereafter show a decreasing response to irradiation into the first days of neonatal life. By giving pregnant female rats 200 rad of whole body irradiation on the twentieth day of gestation, all germ cells in male fetuses are destroyed or lethally injured so that they degenerate when they attempt the definitive postnatal division. This results in a sterile testis.[10, 11] On the other hand, the Sertoli cells and the interstitial cells are not adversely affected by such a low dose of irradiation and continue their normal course of development in the germ cell-free environment.[12]

We obtained timed pregnant Lewis female inbred rats. At the twentieth day of gestation these pregnant rats were anesthetized with ether and treated with 200 rad of whole body irradiation from a cobalt source in the supine position to assure uniform exposure of all in utero animals. The newborn rats were weaned from their mothers at 26 days of age and subsequently were sexed and divided up for the experiment. At 12 weeks of age the male irradiated rats, along with age matched nonirradiated controls, were parathyroidectomized. After one week, when a hypoparathyroid state had been documented, parathyroid allografts were implanted into the testis and beneath the renal capsule in normal male controls, and into the rats who had received in utero irradiation.

Measurement of parathyroid graft survival. Graft survival was determined by weekly measurements of the fasting serum calcium level, which is within the normal range (7.5 to 9 mg%) in rats with surviving parathyroid grafts. The serum calcium determinations were made using a 940 Dow Corning calcium analyzer in our lab. Fasting serum calcium levels below 7.0 mgs% are not seen in euparathyroid rats. Serum calcium levels below 7 mgs% were interpreted as indicating graft rejection. All grafts were harvested after rejection had occurred in nonprivileged sites (beneath the capsule of the kidney). One week after the parathyroid grafts were harvested, serum calcium levels were again determined to ensure that normal calcium levels were not secondary to accessory parathyroid tissue. The

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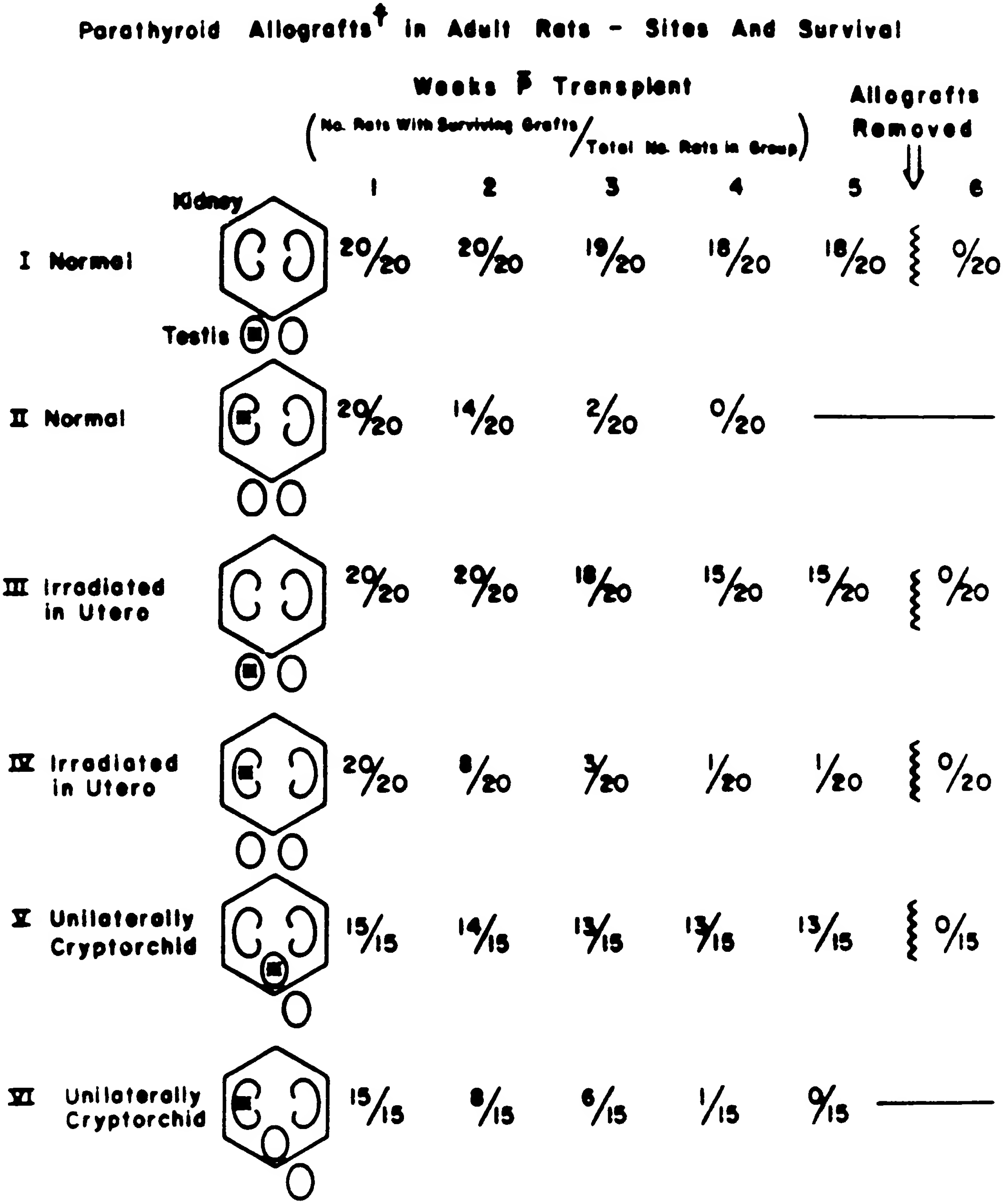

FIG. 1. † Five additional matched animals in each group had isografts which survived uniformly well for the duration of the experiments. * indicates site of parathyroid graft.

data are summarized in figure 1.

Histology. Histology of the cryptorchid and the Sertoli cell-only testes was obtained following preservation and fixation in glutaraldehyde plus osmium. The whole testis was then embedded in plastic and sections 1 micron thick were obtained and stained with toluidine blue to obtain optimal resolution. Also, histology of viable parathyroid allografts removed from the testes of each group was obtained at random.

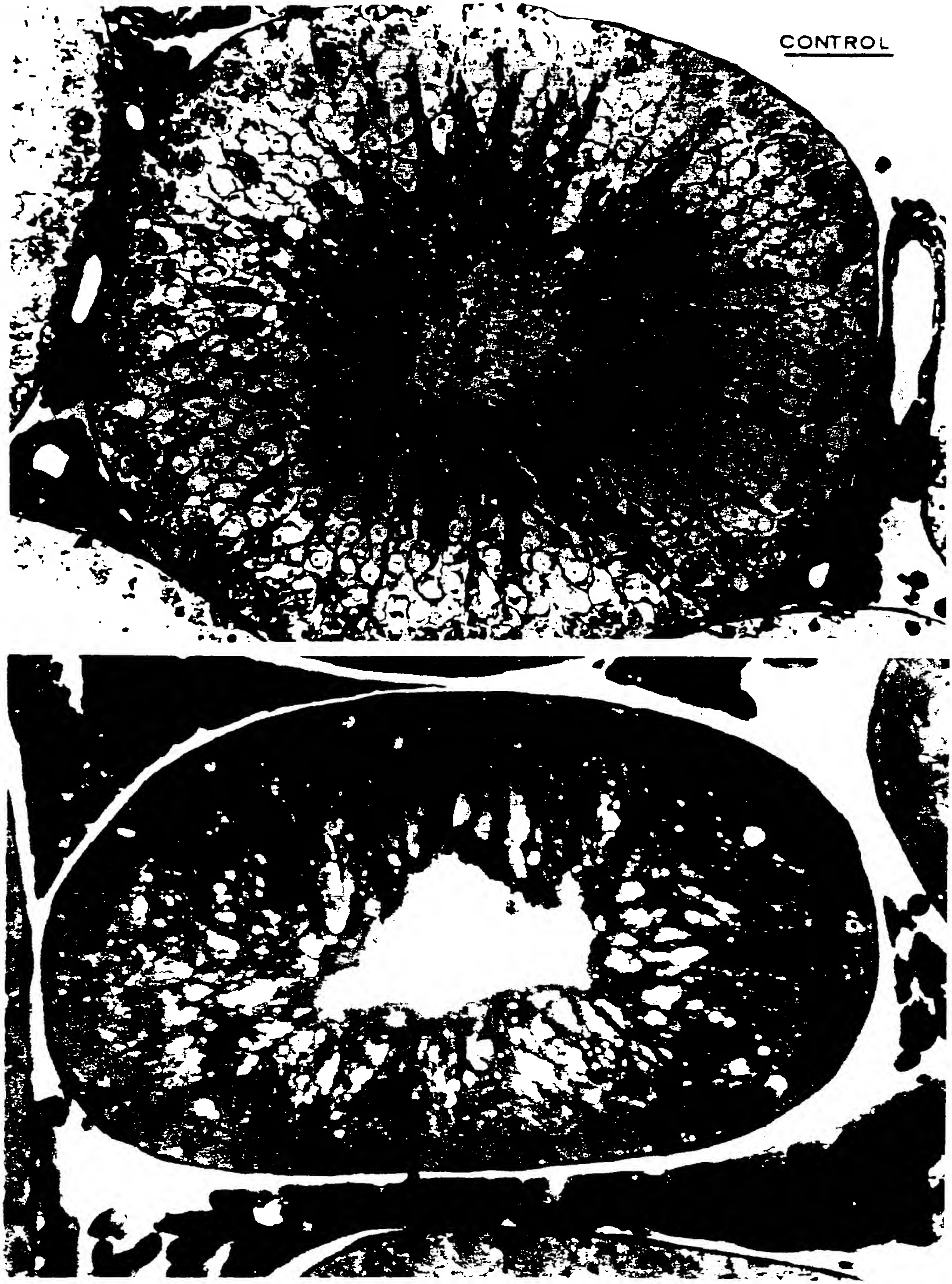

FIG. 2. *A*, section of seminiferous tubule and interstitial cells of normal adult rat.
B, similar section of seminiferous tubule and interstitial cells of rat treated with in utero irradiation, showing exuberant Sertoli cells with complete absence of germinal epithelium and normal interstitial cells.

RESULTS

Adolescent unilateral cryptorchidism abrogated spermatogenesis in the cryptorchid testis, yet preserved the normal hormonal physiology of a fertile rat. Histology of these testes showed atrophy of the germinal epithilium and the absence of spermatogenesis.

Rats irradiated in utero were noticeably smaller and scrawnier then their normal counterparts. However, their ability to reject allografts was not noticeably impaired. Examination of their testes showed them to be about one-half normal size. Histologically there was complete absence of germ cells and spermatogenesis. The Sertoli cells and interstitial cells appeared healthy, justifying their categorization as Sertoli cell only (fig. 2*B*).

Survival of parathyroid autografts was uniformly excellent in all sites in all animals. Parathyroid allografts placed within the testis, as demonstrated by serum calcium levels and by histology (figure 1, Groups I, III, V) showed minimal immune rejection at five weeks. Identical allografts placed beneath the renal capsule in similarly prepared rats (figure 1, Groups II, IV, VI) exhibited a high rate of rejection by four weeks. The advantage in survival between any of the groups with intratesticular grafts and the control renal subcapsular group was highly significant ($p < .005$).

DISCUSSION

The immunological privileged status of the testicle has been established by experiments demonstrating the prolonged survival of skin, parathyroid and other endocrine tissues after transplantation into it.[1-4] These same tissues regularly provoke a rapid and lethal immunologic rejection when transplanted into conventional sites such as beneath the renal capsule, or subcutaneously.[5] The brain, the anterior chamber of the eye and the hamster cheek pouch are other well-known privileged sites;[2,13-15] however, their mechanism for abrogating the immune response differs from that of the testis. The best explanation for the existence of these immunologic sanctuaries has been the lack of a normal lymphatic drainage system.[2,9,15] This anatomic exclusion is thought to result in an afferent immune blockade that effectively hides the transplanted tissue from the normal sensitizing mechanism required for a first set immune rejection. The testis is an exceptional and unique privileged site because it does not share this common anatomy.[9,16,17] It has an easily demonstrated rich lymphatic network that negates the hypothesis of afferent immune blockade. Indeed, many studies have shown that the immune system readily responds with both a humoral and cellular response to antigens exposed within the testis.[16,31] The testis, therefore, has to have another mechanism for blocking immune rejection. Both in vivo and in vitro immunologic studies have attempted to find out how this is achieved.[9,16,21] The hypothesis of this study is that spermatogenesis or the presence of germ cells is a requirement for the existence of this phenomenon.

We have tried to halt spermatogenesis by unilateral cryptorchidism in immature rats and in-utero irradiation, and to eliminate germ cells with in-utero irradiation. By the criteria of light microscopy with optimum technique, in-utero irradiation was effective in achieving these changes in the testes for the duration of the experiment. Since our results indicate that parathyroid allograft survival in the cryptorchid testis with absent spermatogenesis and in our Sertoli cell-only testis is as good as that seen in the normal testes of healthy animals, we conclude that our working hypothesis is wrong. The absence of active spermatogenesis or even the primordial germ cells, as effected by our manipulations, does not alter the immunologically privileged status of the testis in rats as judged by allograft survival. Also, we have reconfirmed that the whole testis is privileged, not only the part protected by the so-called "blood testis barrier." Given the data from earlier experiments indicating testicular steroid production and temperature also were not essential ingredients,[9,32] the number of hypotheses remaining is limited. The Sertoli cell is the only unique component of the testis which we have not eliminated or inactivated in the course of our experiments. It is thought to perform many functions necessary for spermatogenesis, including 1) the formation of the blood-testis barrier and creating the microenvironment necessary for germ cell differentiation,[22,23] 2) acting as the target cell through which FSH and testosterone influence spermatogenesis,[24] 3) the secretion of tubular fluid, 4) the production of testicular androgen binding protein[25,27] and 5) production of inhibin.[28] Also, in the rat, Sertoli cells have been shown to produce an apparently unique polypeptide growth factor.[29] Given this picture, it is possible, even likely that the Sertoli cell also influences the local environment immunologically. The existence of an immunosuppressive factor is suggested not only by our work but by other in vivo and in vitro studies showing the markedly immunosuppressive effects of seminal plasma.[20,21,30]

With this background, we propose the existence of an immunosuppressive factor produced in the testis by the Sertoli cell. This factor and the blood-testis barrier formed by the Sertoli cell act as a dual defense against the induction of autoimmune infertility. Presumably this is why it is extremely difficult to demonstrate autoimmune infertility in men, even in the presence of a variety of antisperm antibodies,[7] and why experimental models of immune infertility have proven difficult to establish.[22]

In summary, the immunological mechanism(s) which make the testis a sanctuary for foreign tissue antigens remains a mystery. There is good experimental evidence which suggests the presence of an active process which incidentally permits privileged allograft survival but which exists teleologically to protect the developing sperm from autoimmune attack. This is in addition to the passive anatomical separation provided by the blood-testis barrier. Our cumulative data strongly implicates the Sertoli cell in this process.

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EXHIBIT E

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Suppression of lymphocyte proliferation by proteins secreted by cultured Sertoli cells

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Summary pd. 10/1988 p. 27-40 14

Secreted proteins from cultured rat Sertoli cells were assessed for effects on phytolectin-stimulated rat splenic lymphocytes. Sertoli cell proteins (SCP) suppressed DNA, RNA and protein synthesis in stimulated rat splenic lymphocytes whether added at 0, 4, 24 and 48 h after culture initiation. SCP preparations were not toxic to cells. SCP suppressive activity was heat stable but was not associated with the carbohydrate component of SCP preparations. SCP also suppressed the proliferation of lymphoid and non-lymphoid cell lines from several different animal species but did not inhibit proliferation-independent lysis of YAC-1 target cells by rat natural killer cells. These results suggest that Sertoli cells synthesize inhibitory factors that might be secreted into seminal plasma. Furthermore, our results demonstrate that one mode of action of these factors is suppression of cell proliferation.

Key words: *Sertoli cells; secreted proteins; immunosuppression; lymphocyte proliferation.*

Introduction

Semen components such as sperm and seminal fluid have the capacity to significantly alter immune responses and could contribute to the

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pathogenesis of a number of sexually transmitted diseases, including acquired immunodeficiency syndrome (AIDS) (Rodman et al., 1985). Sperm can activate complement (Witkin, et al., 1983), suppress several cell-mediated immune functions such as natural killer (NK) cell activity, mixed lymphocyte reactivity, and cytotoxic T lymphocyte generation (Hurtenbach and Shearer, 1982), and inhibit spontaneous and phytolectin-induced T lymphocyte proliferation (Marcus et al., 1978). Seminal fluid has numerous immunosuppressive effects including inhibition of phytolectin-induced T lymphocyte proliferation (Majumdar et al., 1982; Stites and Erickson, 1975), depression of primary and secondary humoral responses (Anderson and Tarter, 1982), and suppression of NK cell, macrophage and neutrophil activity (reviewed by James and Hargreave, 1984). The prostate secretes some of the proteins found in seminal fluid. Prostatic secretions have been shown to inhibit granulocyte function (Stankova et al., 1976) and suppress the response of T lymphocytes to phytolectin stimulation (Mukeherjee et al., 1983).

Sertoli cells are epithelial cells within the seminiferous tubules which synthesize and secrete proteins thought to be essential to spermatogenesis (Kissinger et al., 1982). Sertoli cells also secrete some of the proteins found in seminal fluid. One or more of these proteins may be responsible for some of the immunosuppressive properties observed in seminal fluid preparations. Rat Sertoli cell secreted proteins that have been characterized include androgen binding protein (Fritz et al., 1974; Hagenas et al., 1975), plasminogen activator (Lacroix et al., 1977), ceruloplasmin and transferrin (Skinner and Griswold, 1980; Sylvester and Griswold, 1984). Two sulfated glycoproteins (SGP), called SGP-1 and SGP-2, have been isolated and characterized (Sylvester et al., 1984; Morales et al., 1987; Collard and Griswold, 1987). Their biological activities are still unknown; however, it is suspected that they may be involved in lipid transport. Two heat-sensitive proteins secreted from cultured rat Sertoli cells have been shown to have mitogenic activity for confluent monolayers of certain non-lymphoid cell lines such as BALB/c 3T3 fibroblasts (reviewed by Griswold et al., 1988; Feig et al., 1980; Feig et al., 1983; Holmes et al., 1986) and Dunning prostate R3327H adenocarcinoma cells (Holmes et al., 1986). These proteins were not tested on lymphoid cells. Here we present evidence that one or more of the Sertoli cell secreted proteins are capable of inhibiting phytolectin-induced lymphocyte proliferation in vitro.

Materials and methods

Sertoli cell culture

Sertoli cells from 20-day-old rats were prepared and cultured in serum-

free medium as previously described (Dorrington and Fritz, 1975; Wilson and Griswold, 1979). Prepared cultures were generally 95% Sertoli cells with the remaining percentage consisting of myoid cells and fibroblasts (Wilson and Griswold, 1979). Cells were plated onto 60-mm Falcon dishes and maintained at 32°C in Ham's F-12 medium supplemented with testosterone (0.7 μM) and insulin (5.0 μg/ml). Spent medium from these cultures was collected every 2 days after the initial 2-day incubation period, centrifuged to remove debris, and stored at −20°C. ^{35}S-methionine-labeled secreted proteins were obtained by adding 500 mCi of ^{35}S-methionine to cells which were incubated in Ham's F-12 medium lacking methionine. The total protein concentration of the spent medium was approximately 10 μg/ml.

Lymphocyte preparation

Spleens were aseptically removed from adult, female Sprague—Dawley rats. Single cell suspensions were prepared by perfusion of the intact spleens with Hank's balanced salt solution (HBSS) containing 20% acid citrate dextrose (ACD) as anticoagulant. The perfused spleens were then pressed through a wire mesh screen and the resulting cell suspensions pooled. The cells were centrifuged at 400 × *g* for 10 min and the pellets resuspended in HBSS containing 20% ACD. The centrifugation was repeated and the cells were resuspended in RPMI-1640 containing antibiotics and then counted. For NK activity assays, splenocytes were fractionated on Percoll gradients as previously described (Timonen and Saksela, 1980; Magnuson et al., 1987), and the fraction containing NK activity was used.

Lymphocyte proliferation assays

Splenic lymphocyte preparations were suspended in 1×10^6 per ml in RPMI containing 2.5% normal rabbit serum (NRS) and dispensed into 96-well U-bottom culture plates in 180 μl/well. Optimal concentrations of concanavalin A (Con A, 0.5 μg/ml), phytohemagglutinin P (PHA-P, 25 μg/ml), pokeweed mitogen (PWM, 2.5 μg/ml), or *E. coli* bacterial lipopolysaccharide (LPS, 50 μg/ml) were added to triplicate wells to induce lymphocyte proliferation. Cultures were incubated at 37°C for 64 h, then 1 μCi/well of [^{3}H]thymidine, [^{3}H]uridine, or [^{14}C]leucine was added and the cultures incubated for an additional 8 h. The samples were collected and processed for liquid scintillation counting.

Sertoli cell protein (SCP) preparation

SCP was prepared as previously described (Griswold et al., 1986). Briefly, thawed spent medium was concentrated over Amicon YM-10

ultrafilters and desalted by passage over a Bio-Rad P-6 gel filtration column. The fractions containing the secreted proteins were pooled, lyophilized and stored at −20°C. Lyophilized SCP was weighed and resuspended in HBSS at 3.5—4.0 mg/ml. Samples were sterilized by filtration through a 0.45 μm pore filter and concentrations rechecked by a protein assay (Bio-Rad, Richmond, CA).

Heat treatment

SCP at 3.5—4.0 mg/ml HBSS was heated for 30 min at 56°C in a waterbath or for 3 min in a boiling waterbath. Heat-treated SCP was then diluted in HBSS and added to lymphocyte proliferation assays.

Pronase treatment

A sample of SCP was digested with Pronase (Calbiochem, San Diego, CA) as previously described (Finne and Krusius, 1982). Briefly, a sample of SCP with an internal standard of ^{35}S-methionine-labeled SCP was added to a pre-incubated solution containing incubation buffer (0.1 M Tris—HCl, pH 8.0, 1 mM $CaCl_2$), toluene and pronase. This mixture was incubated at 60°C for a total of 48 h, with additional pronase added after 24 h. The reaction was stopped by heating in a boiling waterbath for 10 min and the resulting mixture was then centrifuged at low speed. The supernatant was removed, passed over a Bio-Rad P-2 gel filtration column and then eluted with 0.1 M pyridine acetate (pH 5.0). The fractions were monitored for radioactivity by scintillation counting and for carbohydrate by the relative absorbance at 490 nm obtained from a phenol-sulfuric acid assay (DuBois et al., 1956). The fractions containing carbohydrate were pooled, lyophilized and stored at −20°C. BSA was subjected to pronase treatment and used as a control.

Suppression of lymphocyte proliferation

SCP was added in 20 μl/well to triplicate wells of a lymphocyte proliferation assay at final concentrations of between 5 and 400 μg/ml. This range of concentrations was reported to be within the physiological total protein concentration in seminiferous tubule fluid (Setchell, 1978). Times of addition were 0, 4, 24 or 48 h after phytolectin addition.

Natural killer (NK) cell activity

^{51}Cr-labeled YAC-1 targets at 1×10^5 per ml RPMI were dispensed in triplicate in wells of a 96-well V-bottom culture plate. Splenic lymphocytes were added in equal volume of RPMI to give effector/target (E : T) ratios of 10 : 1, 25 : 1 and 50 : 1. To test for suspension of effector function, SCP was added at a final concentration of 400 μg/ml to each well.

The cultures were incubated for 4 h at 37 °C, supernatants were collected and the radioactivity determined by gamma counting. The percentage killing was calculated as follows

$$\% \text{ killing} = \frac{(\text{cpm experimental} - \text{cpm spontaneous})}{(\text{cpm total} - \text{cpm spontaneous})} \times 100$$

Spontaneous release was determined by incubating the targets with media alone. The total release was determined by incubating the targets with 1% Triton X-100.

Viability

Viability of SCP-treated cultures was determined after 48 h culture by replacing 100 μl of medium with an equal volume of 0.2% Trypan blue and differentially counting the cells which excluded the dye. Two hundred cells were counted and viability was expressed as a percent of the total number of cells counted.

Suppression of cultured cell lines

A mouse IL-2-dependent T cell line (HT-2), a bat lung fibroblast line (Tb1Lu), a human erythroblastoid cell line (K562), a mouse macrophage-like cell line (P388D1), and a bovine kidney epithelial cell line (MDBK) in growth phase were subcultured in triplicate wells with or without SCP in RPMI or Dulbecco's minimal essential medium containing 10% fetal bovine serum. Cultures were allowed to proliferate at 37°C for 24 h, then [^{3}H]thymidine was added for 6 h and the cultures were collected and processed for scintillation counting.

Results

Inhibition of lymphocyte proliferation

SCP inhibited the response of lymphocytes to the T cell stimulatory phytolectins Con A, PHA and PWM, and to the B cell polyclonal activator LPS in a dose-dependent manner (Fig. 1). At the highest SCP dose, thymidine uptake was lower than seen in unstimulated cultures. Similar results were obtained with the 3 different SCP preparations.

Viability

To determine whether the suppression was due to toxicity of SCP for lymphocyte cultures, Trypan blue was added to 48 h cultures and viability determined (Table 1). The number of cells recovered from each culture was also determined (data not shown) and was found to be comparable in

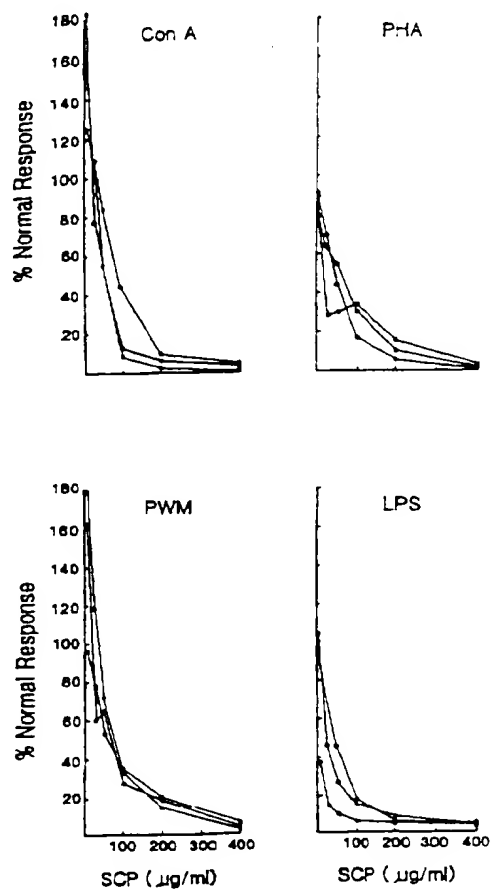


Fig. 1. Inhibition by SCP of the proliferative response of splenocytes to mitogens. SCP was added to cultures of splenocytes containing optimal concentrations of Con A, PHA, PWM or LPS. Each panel represents the effect of adding increasing amounts of 3 different preparations of SCP. The results are expressed as % of the response of splenocytes to mitogen in the absence of SCP. Each point represents the average of triplicate samples. Mean and 1 standard deviation for stimulated and unstimulated cpm in the absence of SCP in 3 different experiments are: 82,740 ± 30,896 (Con A); 122,096 ± 80,582 (PHA); 18,604 ± 4386 (PWM); 3223 ± 599 (LPS); 527 ± 182 (RPMI).

TABLE 1

Viability of SCP-treated splenic lymphocyte cultures.

Additions	% Viability[a]
No additions	95.1
Con A	97.0
Con A + 5 μg/ml SCP	94.4
Con A + 25 μg/ml SCP	96.7
Con A + 50 μg/ml SCP	94.9
Con A + 200 μg/ml SCP	93.0

[a]% viability represents the average % viability from duplicate wells.

all cultures. The results indicate that SCP is neither toxic nor lytic to splenic lymphocytes at any concentration tested.

Effect of delayed addition of SCP to Con A-induced lymphocyte activation

To determine at what stage in the activation of lymphocytes SCP exerts an inhibitory effect, the inhibitor preparation was added at various times after initiation of stimulation (Fig. 2). No difference in suppression was seen when SCP was added at 4, 24 or 48 h after addition of Con A.

Effect on RNA and protein synthesis

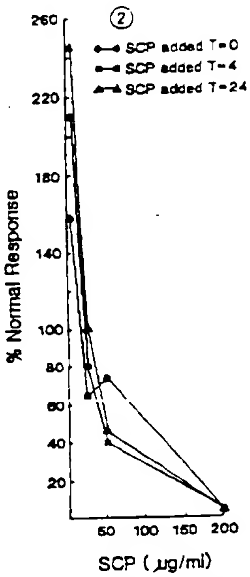
To determine whether SCP inhibited Con A-induced RNA or protein synthesis, cultures stimulated with Con A to which SCP was added upon culture initiation were treated with [^{3}H]uridine or with [^{14}C]leucine and compared to cultures to which [^{3}H]thymidine was added (Fig. 3). SCP appeared to be less inhibitory to RNA synthesis than to either protein or DNA synthesis, although all were suppressed in a dose-dependent manner.

Effect of heat treatment on inhibition by SCP

To determine whether the inhibitory activity of SCP could be eliminated by exposure to heat, SCP was heated at 56°C for 30 min or boiled for 3 min (Fig. 4). Heat treatment did not inactivate SCP.

Effect of pronase treatment on inhibition by SCP

To determine if the inhibitory activity of SCP was due to a protein moiety rather than to complex carbohydrates, a sample of SCP was digested with pronase. The remaining carbohydrate component was isolated and its activity compared with untreated SCP (Fig. 5). The carbohydrate component of SCP did not contain the suppressive activity indicating that suppression of lymphocyte proliferation is a property belonging to the protein component of SCP.



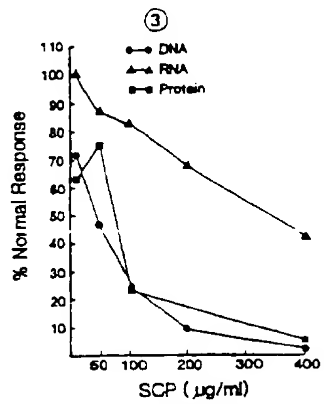


Fig. 2. Effect of delayed addition of SCP on the proliferative response of Con A-stimulated splenocytes. SCP was added at culture initiation ($T = 0$), after 4 h ($T = 4$), or after 24 h ($T = 24$) of culture. Results are expressed as a % of the response of splenocytes to Con A in the absence of SCP. Each point was derived from the average cpm of triplicate samples. The experiment was repeated with a different SCP preparation with similar results.

Fig. 3. Effect of SCP on DNA, RNA and protein synthesis in Con A-stimulated splenocytes. SCP was added to Con A stimulated splenocytes and the cultures were assessed for DNA, RNA or protein synthesis by the addition of [^{3}H]thymidine, [^{3}H]uridine, or [^{14}C]leucine, respectively. The results are expressed as a % of the response to Con A in the absence of SCP. Each point was derived from the average cpm of triplicate samples. The experiment was repeated with two different SCP preparations with similar results.

Effect of SCP on proliferation of cell lines

Because SCP inhibited lectin-induced lymphocyte proliferation, the preparation was tested on cell lines of lymphoid and non-lymphoid origin to determine if the inhibitory activity was specific for lymphocytes (Fig. 6). SCP inhibited the proliferation of all lines tested in a dose-dependent manner. Interestingly, its effects on the T cell line, HT-2, with suppression at high doses and apparent stimulation at low doses, were similar to those observed with some SCP preparations on phytolectin-stimulated splenic lymphocytes.

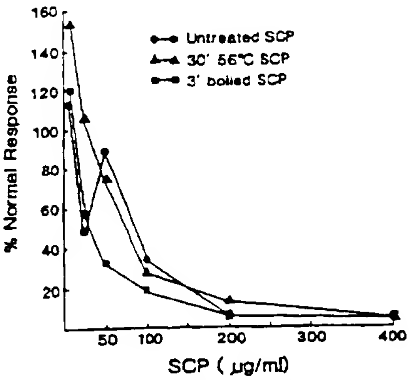


Fig. 4. Effect of heat treatment on suppression of Con A-stimulated splenocytes by SCP. SCP which was untreated, heated at 56°C for 30 min, or boiled for 3 min was added to Con A stimulated splenocytes at culture initiation. Results are expressed as a % of the response of splenocytes to Con A in the absence of SCP. Each point was derived from the average cpm of triplicate samples. The experiment was repeated with a different SCP preparation with similar results.

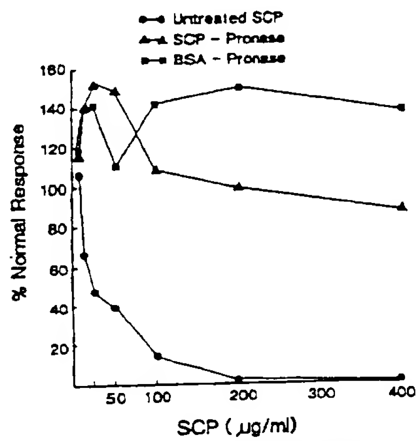


Fig. 5. Effect of pronase treatment on SCP-induced suppression of Con A-induced splenocyte proliferation. Untreated SCP, pronase-treated SCP, or pronase-treated BSA was added to Con A-stimulated splenocytes at culture initiation. Results are expressed as a % of the response of splenocytes to Con A in the absence of SCP. Each point was derived from the average cpm of triplicate samples. A similar experiment using a different SCP preparation gave similar results.

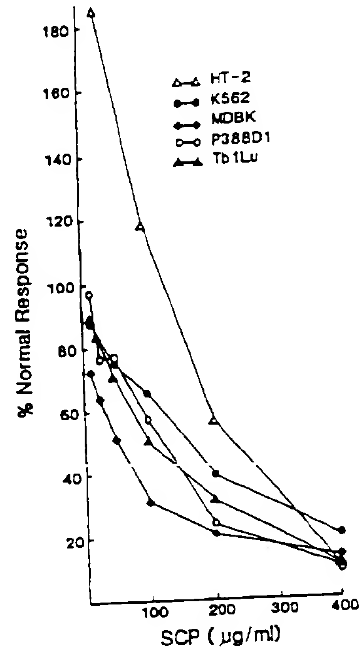


Fig. 6. Inhibition of proliferation of cell lines by SCP. SCP was added to cultures of HT-2, K562, MDBK, P388D1, or Tb1Lu cells and [^{3}H]thymidine incorporation measured. Results are expressed as a % of cell proliferation in the absence of SCP. Each point was derived from the average cpm of triplicate samples. Each cell line was tested in two separate experiments with similar results.

Effect of SCP on NK cell activity

NK cell activity is not dependent on cell proliferation. Therefore, the ability of rat splenic lymphocyte suspensions to lyse the NK cell sensitive target cell line YAC-1 was used to determine whether SCP was inhibitory to proliferation-independent effector cell function (Table 2). The results from two separate experiments indicate that SCP does not suppress NK cell activity.

TABLE 2

Effect of SCP on NK cell activity of rat splenocytes.

E : T ratio	SCP[a]	% killing[b]
Experiment 1		
10 : 1	−	13.6
10 : 1	+	9.3
25 : 1	−	27.6
25 : 1	+	27.6
50 : 1	−	39.6
50 : 1	+	32.9
Experiment 2		
10 : 1	−	9.1
10 : 1	+	9.5
25 : 1	−	13.1
25 : 1	+	18.6
50 : 1	−	19.1
50 : 1	+	33.9

[a] Presence or absence of SCP at 400 μg/ml.

[b] Determined from average cpm for triplicate wells.

Discussion

In this study we used a preparation of secreted proteins synthesized by Sertoli cells in culture to assess the effect of Sertoli cell secreted products on rat lymphocyte proliferation in vitro. We found that SCP inhibited mitogen-induced lymphocyte proliferation in a dose-dependent manner whether added at culture initiation or up to 48 h after culture initiation. Sertoli cell culture medium not previously exposed to Sertoli cells was not inhibitory (data not shown). DNA and protein synthesis were inhibited to a greater extent than was RNA synthesis by SCP. These SCP inhibitory preparations, however, were not toxic to activated lymphocytes at any concentration tested, and inhibitory activity could be removed by washing the cells within the first 24 h of culture (C.R. Wyatt, unpublished observations). Inhibitory activity was heat-stable and not associated with carbohydrate moieties. In addition, SCP inhibited proliferation of several tissue culture lines, including non-lymphoid cell types, but did not inhibit proliferation-independent NK cell activity.

Several conclusions can be drawn from these observations. The first is that cultured Sertoli cells produce one or more proteins which can inhibit lymphocyte proliferation. This observation is consistent with studies using crude seminal plasma in which inhibition of responses to T cell

phytolectins have been reported (Majumdar et al., 1982; Anderson and Tarter, 1982; Stites and Erickson, 1975). Our studies indicate that Sertoli cells synthesize inhibitory factors which could be secreted into seminal plasma, and might inhibit the de novo development of immune responses, such as responses to sperm antigens.

The second conclusion is that the inhibition appears to involve a late event in lymphocyte proliferation. This contention is based on the observation that SCP is suppressive when added to mitogen-stimulated splenocytes at any time within the first 48 h. Because RNA synthesis is less inhibited than protein or DNA synthesis by SCP, it suggests that translation of mRNA might not be occurring.

The third conclusion is that SCP preparations do not interfere with established effector cell function. NK cell activity does not depend on cell division, adding support to the idea that inhibition by SCP is associated with cell proliferation. Furthermore, our results indicate that Sertoli cells do not produce the NK cell inhibitory activity reported to be present in seminal plasma (Marcus et al., 1985).

The fourth conclusion is that the mechanism of SCP suppression of lymphocyte proliferation might involve a general event occurring in all dividing cells. It does not appear to be related specifically to lymphocytes. The observation that SCP inhibits proliferation of non-lymphoid cells in growth phase from several sources indicates that SCP might function at a metabolic site common to all cells. In addition, the fact that the cell lines were derived from animal species as diverse as mice, bats and cattle suggests that the inhibitory mechanism is not species-specific.

SCP preparations consist of at least 7 major and many minor glycoproteins of which there might be more than one molecule with an effect on lymphocytes. Some preparations of SCP appear to show a slight enhancement of Con A- and PWM-induced lymphocyte activation when added at low concentrations (25 μg/ml final concentration). Similarly, low concentrations of some preparations of SCP enhance proliferation of an IL-2-dependent T lymphocyte line but have no enhancing effect on any of the non-lymphoid cell lines tested. We have not yet isolated the apparent mitogenic factor and do not know why it does not appear to be present in all Sertoli cell supernatants. However, we speculate that, at high SCP concentrations, the inhibitory activity is SCP preparations may be capable of overcoming the mitogenic activity. Upon dilution, the inhibitory activity may be diluted away sooner leaving only the mitogenic activity. Further analysis of any mitogenic molecule secreted by Sertoli cells will require purification of the factor from the crude SCP.

The SCP inhibitor of cell proliferation discussed here has not yet been purified to homogeneity. However, preliminary results suggest that the inhibitory activity lies within a fraction containing proteins with apparent

molecular weights in the range of 10,000—25,000. These molecules are too large for any of the known suppressive molecules such as prostaglandins. Purification of the suppressive protein(s) is presently underway.

We believe that Sertoli cells produce SCP. We know nothing as yet about the role of hormones in the induction of the suppressive factor, nor do we understand what function it might have in regulation of sperm maturation. When we have purified and sequenced the factor, we can make the necessary reagents to study it further. We believe that such reagents will be required to confirm the Sertoli cell specific origin of the factor and better understand its role in the male reproductive system.

Seminal plasma has been reported to inhibit a wide variety of immune system functions (James and Hargreave, 1984). We have shown here that cultured Sertoli cells might contribute at least one of the inhibitory effects observed in seminal fluid. Our culture system should be of value in determining which of the other immunomodulatory effects of seminal fluid can be attributed to Sertoli cell secretions.

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EXHIBIT F

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TRANSPLANTATION

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SUCCESSFUL ISLET/ABDOMINAL TESTIS TRANSPLANTATION DOES NOT REQUIRE LEYDIG CELLS[1]

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Pancreatic islet allo- and xenografts are not rejected and exhibit long-term β-cell function if transplanted into the abdominal testis of the diabetic host. Successful transplantation appears dependent on local factors unique to the abdominal testis. Because Leydig cells remain viable in abdominal testes, which also retain high levels of testosterone, the following question was addressed: do Leydig cells and/or their secretory products influence islet transplantability in the successful islet/abdominal testis transplantation model?

Streptozotocin-induced diabetic rats (Sprague-Dawley) were injected with 75 mg/kg ethane dimethanesulfonate (EDS) to selectively eliminate Leydig cells prior to or following transplantation with islets isolated from the BBWOR[dr] rat. Subcutaneous silastic tubes packed with estradiol prevented Leydig cell repopulation in the EDS-treated recipient.

Grafted diabetic animals, including the EDS-treated rats with serum testosterone at castration levels, became nornoglycemic following islet transplantation and remained so for up to ten months. Leydig cells were not observed in testes of the EDS- or EDS/estradiol-treated rats, whereas the transplanted islets within these testes appeared structurally normal and highly vascularized. Islets resided within the testicular interstitial compartment and contained α-, β and δ-cells, as identified by electron microscopy. Beta cells were most prominent, contained secretory granules and exhibited a close structural and functional relationship with adjacent intraislet capillaries.

We conclude that Leydig cells and Leydig cell secretory products, including testosterone, are not necessary for protecting islets against rejection and they do not play an obligatory role in the success of the islet/abdominal testis transplantation protocol. Leydig cells and Leydig cell secretory products do not promote long-term β-cell function and are not required for the return to and maintenance of normoglycemia in the grafted diabetic rat.

It is well known that the testis is an immunologically privileged site that provides a unique environment for enhanced transplantation success (*1–7*). However, mechanisms by which this phenomenon is affected are, as yet, undefined. Selawry and coworkers have shown that the abdominal testis, rather than the testis in its original scrotal position, is the most suitable site for extended functional survival of islet allo- and xenografts (*8, 9*).

It has been suggested that the elevated levels of intratesticular testosterone and/or progesterone, products of the testicular interstitial cells of Leydig, may cause an inhibition of the local immune response (*10–12*) and therefore provide the testis with its unique immunologically privileged status. In a previous study we showed that the administration of leuprolide, a GnRH analog, resulted in a significant decrease of serum and intratesticular testosterone levels, but had no effect on the functional survival of islet allografts in the nonimmunosuppressed recipient (*13*). However, steroidogenesis remained active as evidenced by a significant elevation of serum progesterone (*13*). Hypophysectomy ablated both of these steroids (*13*) but may not have interfered with the secretion of nonsteroidal secretory products of the Leydig cell. Nonsteroidal Leydig cell products, including activin and inhibin, have been postulated as being factors which may play a role in an inhibited local immune response (*7, 13*).

The aim of this study was to examine the survival of islet allografts in the abdominal testes of diabetic rats in whom Leydig cell steroidogenesis and other synthetic and secretory functions had been eliminated completely rather than just suppressed. The compound ethane dimethanesulphonate (EDS) is known to selectively destroy Leydig cells after a single intraperitoneal injection reducing testosterone to castration levels (*14*). The concomitant treatment of EDS-treated animals with β-estradiol prevents Leydig cell regeneration, making it possible to determine the transplantability and examine the function of islet transplants in the selective absence of Leydig cells. In this report we describe the effects of Leydig cell depletion on the structural and functional characteristics of grafted islets in normoglycemic rats with established islet allografts and determine if Leydig cells and their secretory products are necessary to establish and/or maintain a successful islet transplant in the abdominal testis of a diabetic rat.

MATERIALS AND METHODS

Animals and metabolic parameters. Male Sprague-Dawley (outbred) rats (250±11 g) were used as recipients of islet implants. Prior to implantation they were made diabetic with a single intravenous injection of streptozotocin (60 mg/kg body weight). Female BBWOR[dr] rats (RT-1[u]) were used as islet donors. Streptozotocin-induced diabetic rats were kept in metabolic cages and metabolic parameters—including body weight, urine volume, and urine glucose content—were measured at daily intervals. Additionally, plasma glucose concentrations were determined at weekly intervals. Only rats with random plasma glucose levels in excess of 400 mg/dl were transplanted.

Islet isolation and incubation. Islets were isolated according to a method described by James et al. (*15*). Briefly, rat pancreases were distended in situ by the injection into the common bile duct of 10–12

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ml ice-cold collagenase solution (Sigma, type XI, 0.5 mg/ml). Distended pancreases were removed and incubated in a stationary water bath for 17 min at 37°C. Following manual disruption for 1 min, digested tissue was washed 2–3 times by centrifugation in cold Hanks' buffer. The resulting tissue/islet suspension was twice layered on Ficoll gradients (*8*) and separated by centrifugation at 500 ×g. The purified islets were collected, washed in Hanks' buffer and transferred in groups of 150 to Petri dishes containing 6 ml CMRL-1066 culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated for 4–7 days at 37°C in 5% CO_2-95% air. The incubation period chosen was a matter of convenience. Islets cultured for two days will suffice.

Islet transplantation. Transplantation of incubated islets into the abdominal testis was performed as previously described in detail (*9*). Briefly, each rat received an intratesticular injection of 10 islets/g of body weight. The grafted testis was then immediately anchored into the abdominal cavity by simple surgical fixation with suture. Rats were not immunosuppressed and no insulin was given. Following surgery, the grafted rats were transferred to metabolic cages for further daily examination of the metabolic parameters as outlined above. A rat was considered "cured" if the following criteria applied: a random plasma glucose level of 130 mg/dl or less, rapid weight gain, aglycosuria, abrupt reversal to hyperglycemia after surgical removal of the grafted testis, and histological confirmation of the presence of viable β-cells within the testis.

Preparation of EDS. This drug was prepared according to the method of Jackson and Jackson (*16*). A mixture of 45 ml of pyridine and 9 g of ethylene glycol was prepared and cooled in an ice-salt bath. To this mixture 34 g of redistilled methanesulphonyl chloride was added drop-wise from a separating funnel while the reaction temperature was being maintained below 10°C. The ice bath was then removed and when the reaction mixture attained room temperature it was added, with stirring, to a prepared ice-acid mixture consisting of a 1-L beaker three-quarters filled with crushed ice and 45 ml of concentrated sulfuric acid. Stirring was continued until the separating oil was solidified. It then was filtered off by suction and washed first with cold water to remove the acid and finally with chilled methanol. Two crystallizations from chloroform-methanol yielded about 15 g of pure ester. The pure ester, insoluble in water, was dissolved first in dimethylsulfoxide (0.5 ml/75 mg). Water (1.5 ml) was added drop-wise with agitation to give the required 2 ml/kg body weight dose volume.

Preparation of β-estradiol implants. Silastic Medical Grade Tubing (Dow Corning 601-335; ID 0.132 inches, OD 0.183 inches) was cut in approximately 2 cm segments, and one end was closed with Silastic Medical Brand Adhesive Silicone Type A (Dow Corning) and allowed to dry overnight. The following day about 0.5 cm of β-estradiol (Sigma) was pushed into the tubing and the other end was sealed. After it was allowed to dry for 48 hr, the capsules were soaked in PBS for an additional 48 hr. The capsules were implanted in rats underneath the skin on the dorsum of the neck through a small incision.

Treatment of rats with EDS and β-estradiol. A total of 31 diabetic, hyperglycemic male rats received islet transplants as described above and treated as follows:

- Group 1: Five diabetic rats were used as controls and given an i.p. injection of 2 ml of the solvent, DMSO, prior to transplantation. No other therapy was given.
- Group 2: Six diabetic rats were treated with EDS and transplanted within the next 15 days.
- Group 3: Fourteen diabetic rats first were transplanted and then treated with EDS following an established cure.
- Group 4: Six diabetic rats first were transplanted and then treated concurrently with EDS and β-estradiol following an established cure.

In addition to the collection of the metabolic parameters, blood samples were obtained before and after EDS and EDS/EST treatment to determine serum testosterone levels. For the determination of intratesticular testosterone levels, the grafted testes were cut into 100-mg fragments from which the steroid hormone was extracted with methylene chloride. The extracts were stored at −20°C until assayed for testosterone using a radioimmunoassay kit obtained from New England Nuclear.

Tissue collection and testicular morphology. At specific intervals, ranging between 44 and 437 days after transplantation, a total of 10 rats (groups 1–4) were orchiectomized. Bisected testes were immersion fixed with 5% glutaraldehyde in 0.1M s-Collidine buffer for 1 hr, washed in buffer, and postfixed for an additional hour with 1% osmium tetroxide in 0.1 M buffer. Small tissue fragments (approximately 25 mm²) were dehydrated through a graded series of ethyl alcohols, transferer to propylene oxide and embedded in Epon 812/Araldite plastic resin. Thick (0.5-μm) and thin (900-ng) sections were stained routinely with toluidine Blue and uranil acetate/lead citrate, respectively, for structural analysis by light and electron microscopy.

RESULTS

Metabolic parameters. The mean nonfasting plasma glucose levels in 5 control, DMSO-treated rats and 7 EDS-treated rats were 470 mg/dl ±28 and 433 mg/dl ±34, respectively, prior to transplantation. Following the injection of islets into the abdominal testis, both the control and EDS-treated rats became normoglycemic within 48 hr. The mean nonfasting plasma glucose levels 70 days after transplantation were 111 mg/dl ±9 and 131 mg/dl ±8, respectively. The average weight gain over a 70-day period following transplantation of the 5 age-matched control and of the 7 EDS-treated rats was 110 g ±7 and 100 g ±25, respectively.

Duration of normoglycemia. With the exception of two rats, all grafted animals in groups 1–4 became normoglycemic following islet transplantation and remained normoglycemic for greater than four months or until sacrificed for tissue collection. Two rats in group 2 became hyperglycemic, one in whom the testis had reverted to a scrotal position and one for unexplained reasons.

Serum and intratesticular testosterone. Serum testosterone was determined approximately 30 days following EDS (n=13) and EDS/EST (n=10) treatments (Fig. 1). Mean values for the EDS-treated group (0.09 ng/ml ±0.02) and the EDS/EST-treated group (0.02 ng/ml ±0.02) both were significantly ($P<.05$) lower than mean values for either normal control rats (3.17 ng/ml ±1.09) or untreated diabetic rats with abdominal testes (3.13 ng/ml ±1.6). Intratesticular testosterone was determined in untreated (n=20) and EDS-treated (n=2) diabetic rats with abdominal testes (Fig. 2). The mean value for the EDS-treated group (1.14 ng/100 mg testis weight ±0.2) was significantly ($P<.05$) lower than the control value (26.0 ng/100 mg testis weight ±7.0).

Structural analysis of grafted testes: Group 1. Testes from the untreated, grafted rat were typical of cryptorchid testes and conformed to the description of altered tissue histology in experimentally induced cryptorchid rat testes as described by Clegg (*17*). In general, there was overall seminiferous tubule atrophy, with an increase in the tubule wall thickness (Fig. 3). Tubules were devoid of most germ cell types, showing, occasionally, the presence of spermatogonia in the seminiferous epithelium. Sertoli cells were conspicuous due to the absence of germ cells and appeared normal in structure, with the exception that the highly organized architectural relationship of Sertoli cell cytoplasm with germ cells was nonexistent (Fig. 3). Typically, there was a high density of cells in the testicular interstitial compartment giving the appearance of Leydig cell hyperplasia (Fig. 3). Likewise, there appeared to be an increased amount of interstitial extracellular matrix (Fig. 3).

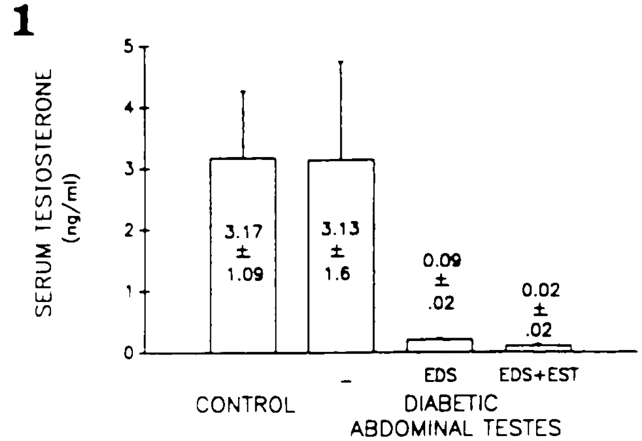

FIGURE 1. Serum testosterone was significantly ($P<.05$) lower in diabetic rats with abdominal testes treated with either EDS or EDS/EST when compared with the levels of serum testosterone in both normal control rats and untreated diabetic rats with abdominal testes.

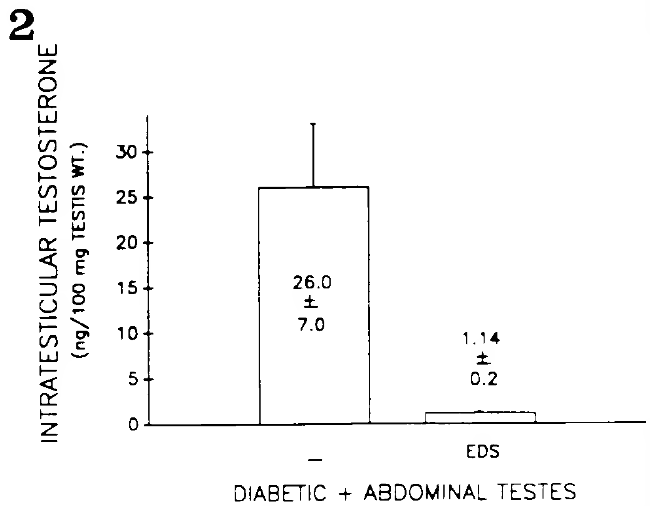

FIGURE 2. Intratesticular testosterone was significantly ($P<.05$) lower in EDS-treated diabetic rats with abdominal testes when compared with intratesticular testosterone in untreated diabetic rats with abdominal testes.

Transplanted islets in testes of group 1 rats appeared similar to islets in situ and resided in the testicular interstitial compartment along with normal appearing testicular macrophages, interstitial Leydig cells, and small blood and lymphatic vessels (Fig. 3). Some islets appeared partially encapsulated by a type I collagen-rich extracellular matrix that contained fibroblast. In most cases, however, islets were directly adjacent to one or more seminiferous tubules without intervening matrix (Fig. 3). Islets had thin connective tissue trabeculae that contained normal appearing capillaries and which separated the endocrine cells into distinct clusters (Fig. 3). Each cluster was composed of large ovoid cells (10–25 nm) with round, centrally located euchromic nuclei (8–10 nm) each containing distinct nucleoli (Fig. 3). Most of the cells contained a variable number of homogenous secretory granules which, by electron microscopic examination, were identified as secretory granules unique to the α-, β-, and δ-cell types (Fig. 3). The structure and dimensions of these secretory granules conformed to the description of such granules found in normal islets as described by Rhodin (*18*). The primary cell type of each cluster was the β-cell, which represented approximately 60–90% of the cell population.

Groups 2–4. Testes from rats treated first with EDS and then grafted (group 2) appeared similar to testes from group 1 animals (Fig. 4). Both the testicular tissue and islet implants

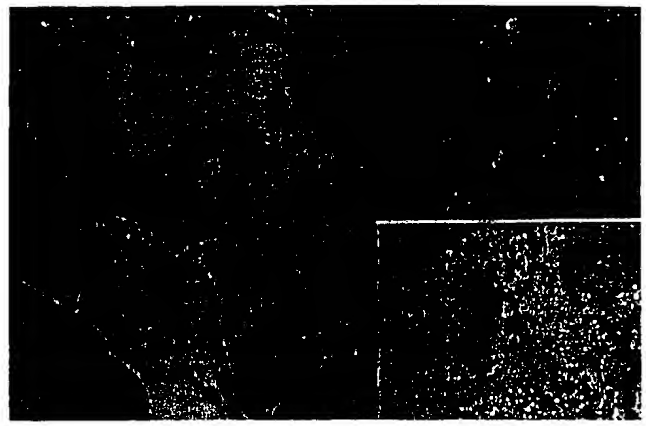

FIGURE 3. This light micrograph illustrates the structural arrangement of a pancreatic islet within the abdominal testis of an untreated diabetic rat. The islet (I) resides within the testicular interstitial compartment and outside of the seminiferous tubule. Islet cells are partially segregated into cell clusters by connective tissue trabeculae that contain intraislet capillaries (curved arrows). The seminiferous tubule has a thickened tubule wall (T) and its seminiferous epithelium is depleted of germ cells. Most of the remaining epithelial cell types are Sertoli cells (S). Also present within the testicular interstitial compartment are numerous Leydig cells (straight arrows) (×1000). Inset: With electron microscopy, cells of the implanted islet are identified as α-cells (A), β-cells (B), and δ-cells (D) by the unique structure of their secretory granules (×1500).

FIGURE 4. This light micrograph shows an implanted islet (I) within the abdominal testis of a diabetic rat treated with EDS. Histology of both the islet and seminiferous tubule is identical to that described for the untreated grafted rat (Fig. 3) with the notable exception that there are no Leydig cells present. Islets are separated into cell clusters by connective tissue trabeculae (curved arrows). The testicular interstitial compartment contains fusiform-like fibroblasts, testicular macrophages the cytoplasm of which is highly vacuolated and filled with dense bodies (straight arrow) but no Leydig cells. (S) Sertoli cells; (T) seminiferous tubule wall (×1100). Inset: The predominant cell type within this implanted islet is the β-cell (B), identified in this electron micrograph by the ultrastructure of its secretory granules (×2500).

conformed to the description of these tissues as described above, with the notable exception that Leydig cells were not present within the testicular interstitial compartment (Fig. 4). Macrophages were typical in their appearance and distinctive because of their highly vacuolated cytoplasm, unlike the dense and fusiform-like fibroblasts. This appearance of testicular tissue and the notable absence of Leydig cells conformed to previous descriptions of testes treated with EDS (*14*). EDS treatment following islet transplantation (group 3) did not alter testicular and islet histology or ultrastructure beyond that described for testes from rats in group 2. Treatment with EDS/EST (group 4) also did not result in testicular and islet morphology different from that described for animals in groups 2 and 3 when structurally analyzed at one or three months following treatment (Fig. 5).

All islet implants observed (groups 1-4) exhibited a close structural relationship to normal-appearing intraislet capillaries (Figs. 3, 4, and 5). Some β-cells contained secretory granules that appeared to have been fused with the cell membrane and in the process of depositing their secretory product into the perivascular space of the adjacent capillary (Fig. 6).

DISCUSSION

Pancreatic islet implants survived and demonstrated normal β-cell function for extended periods—up to ten months—in Leydig cell-depleted abdominal testes of the diabetic rat host. The recipient rat had dramatically reduced serum and intratesticular testosterone, comparable to castration levels, and presumably all other Leydig cell secretory products. Secretory reduction was concurrent with successful transplantation, as determined by the sustained maintenance of normoglycemia in the once severely hyperglycemic rat. Reduction of testosterone either prior to islet transplantation or following an established graft did not interfere with the establishment of normal islet structure within the testis or continuation of normal intratesticular β-cell function.

Although the success of abdominal testis islet transplantation for the cure of diabetes has been demonstrated in both the streptozotocin-induced and BBWOR^{dp} diabetic rat (*9*), this is the first time that successful testicular transplantation of islets, or any tissues, has been documented in testes devoid of Leydig cells. Islet transplantation into testes of hypophysectomized diabetic rats with reduced testosterone has been reported by Selawry et al. (*9*). In this study, however, nonsteroidal secretory products of the Leydig cells, such as activin and inhibin, could not be eliminated as beneficial participants in the successful transplantation protocol. Although there is support for the participation of testosterone as a local suppressant of the immune response in the testis (*10-12*), the present study clearly illustrates that there are no secretory products from Leydig cells that are necessary to either initiate or maintain the normal structure of grafted islets or the normal function of β-cells.

It has long been observed that the testis exhibits a privileged immunological status in that it contains, but does not reject, several highly immunogenic germ cell autoantigens (*19*) and exists as a site of enhanced immunological protection for transplanted tissues (*1-3, 5-7*). The protection of testis autoantigens from the immune system can be explained by their isolation in the immunologically safe adluminal testicular compartment (*20*), sequestered on the safe side of the blood-testes barrier (*21*). Protection from antigens that reside on the vascular side of the blood-testis barrier is not yet understood.

Because the islet implants in this study, and probably all surgical implants in the testis, resided within the interstitial compartment of the testis and not in the seminiferous epithelium, it is misleading to assume that immunological protection of the testicular islets was in some way mediated by the blood-testis barrier. Islets located in the interstitial compartment were exposed to the systemic immune system and not physically protected by the blood-testis barrier, as are the highly antigenic spermatids (*22*). It is clear from the results of this and our previous studies (*9, 23, 24*) that some other local mechanism not involving the blood-testis barrier must provide for immu-

FIGURE 6. This electron micrograph of a β-cell of an islet implanted into the abdominal testis of an EDS/EST-treated diabetic rat contains structurally normal secretory granules (B). One of these granules appears to be fused with the cell membrane and to have released its contents (curved arrow) into the perivascular space of an adjacent intraislet capillary. (N) nucleus; (E) capillary endothelium; (L) lumen of capillary (×30,400).

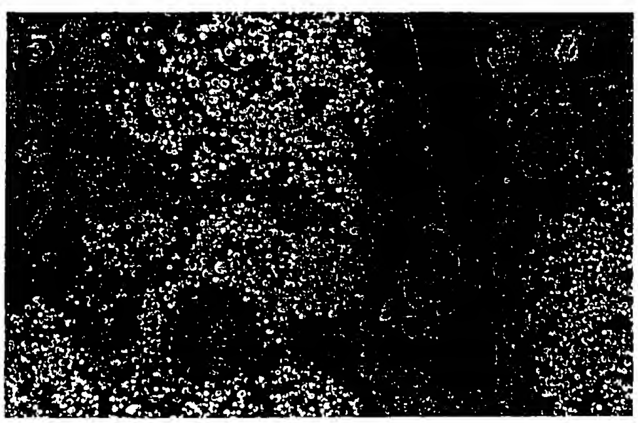

FIGURE 5. EDS/EST treatment had no additional effect on the structure of implanted islets and testicular tissue beyond that described for treatment with EDS alone (Fig. 4). Leydig cells were absent—and, as shown in this electron micrograph, most of the implanted islet cells were β-cells that contained many secretory granules (curved arrows) typical of this cell type. The β-cells (B) are directly adjacent to a seminiferous tubule wall (T) and separated by a connective tissue trabeculum containing an intraislet capillary. RBC: red blood cell within the intraislet capillary (×2000).

nological protection of the islet graft and that this mechanism is heat dependent.

Support for this hypothesis was recently provided by the identification of autoantigens in the mouse testis that were identified and located outside the blood-testis barrier (*25*). Their immunological protection, like the testicular islets, was mediated by some yet-to-be-defined mechanism.

Although the testis has been described as an immunologically suppressed tissue (*7*), the elaborate series of studies of Head and coworkers (*5, 6, 12*) confirmed that the efferent immune responses within the testes is not deficient. It is likely then that local factors other than deficient testicular macrophages provide for the protection of antigenic germ cells outside the blood-testis barrier and interstitial compartment implants.

It is not probable that germ cells are involved, because they are readily depleted in the abdominal testis and because allografts display prolonged survival within testes subjected to experimental cryptorchidism (*12, 26*). Recently, Selawry and coworkers grafted islets into scrotal testes depleted of germ cells by the technique of cold testicular ischemia (*27*). The diabetic rats remained hyperglycemic for thirty days following transplantation, at which time the grafted testes was surgically fixed into the abdomen. Experimental cryptorchidism of the germ cell depleted, grafted testes resulted in a return to normoglycemia within 24 hr (unpublished observations) illustrating both noninvolvement by germ cells and the obligatory role of elevated temperature (e.g., abdominal temperature) in the success of this transplantation protocol.

By the elimination of other cell types, the Sertoli cell becomes the most likely testicular cell type involved in providing the testis with its unique immunological environment. This hypothesis is supported by the recent reports that Sertoli cells produce immunoregulatory proteins (*7, 28*). We are currently evaluating the role of Sertoli cells in successful pancreatic islet transplantation in the diabetic rat testis.

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PRODUCTION OF A FACTOR, OR FACTORS, SUPPRESSING IL-2 PRODUCTION AND T CELL PROLIFERATION BY SERTOLI CELL–ENRICHED PREPARATIONS

A Potential Role for Islet Transplantation in an Immunologically Privileged Site[1]

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Isolated islet allografts survive indefinitely in the abdominal testis of nonimmunosuppressed diabetic rats. The predominant feature of these testes is that the presence of Sertoli cells, but not Leydig cells, is required for extended survival of the islet allografts. Sertoli cells cultures were therefore established in vitro and we examined the effects of the conditioned media on Con A–stimulated spleen lymphocyte proliferation. These studies revealed that a product(s) secreted by Sertoli cells inhibits Con A–stimulated lymphocyte proliferation in a dose-dependent manner. The synthesis of this product is both temperature-dependent, occurring predominantly at 37°C, and hormone-dependent, requiring the presence of follicle stimulating hormone, in the culture medium. We further examined the mechanism of inhibition of lymphocyte proliferation and showed that Sertoli cell–enriched media inhibit the production of IL-2 in a dose-dependent manner. Furthermore, the finding that the addition of exogenous IL-2 is not able to reverse this inhibition indicates that the Sertoli cell–enriched media inhibit both IL-2 production and IL-2 responsiveness of T lymphocytes.

The testis has been considered an immunologically privileged site since the late 1930s when Greene transplanted pieces of human mammary cancer into the testes of rabbits and showed that these cells survived long enough to be examined for response to chemotherapy (*1*). Subsequent studies have shown that other cell types such as skin (*2*), parathyroid (*3*), and pancreatic islets (*4*), also live longer in the testes compared with the survival of identical tissues in organ sites such as the renal subcapsular space or the liver. Studies done over the past five years in our laboratory, have shown for the first time that not only is the testis in its original scrotal position an immunologically privileged site, but that the abdominal testis, in particular, provides an extraordinary safe environment for the extended survival of islet grafts. Long-term survival occurred irrespective of the origin of the isolated islets: thus islets grafted across major histocompatibility barriers (*5*), islet xenografts (*6*), and islets of MHC-compatible donors grafted into rats with spontaneous diabetes mellitus of autoimmune etiology (*7*) continued to function indefinitely in diabetic recipients.

The mechanisms responsible for the remarkable survival of islet grafts in the cryptorchid testis are not known. One or more of the three major cellular components of the testis—including sperm, Leydig cells, and/or Sertoli cells—may contribute to this phenomenon. Sperm and seminal fluid have immunosuppressive effects on phytolectin-induced cell-mediated immune responses (*8*). But since spermatogenesis is rapidly impaired in the abdominal testis it is unlikely that the sperm cell is responsible for immunologic protection. Previous efforts in our laboratory have evaluated the potential immunosuppressive role of the Leydig cell. A variety of techniques were used to selectively destroy local steroidogenesis in the testis (*9*). These studies showed that islet allografts survival was not affected, even in testes devoid of Leydig cells (*10*).

The current study was designed to evaluate the contribution of the Sertoli cell toward producing this immunoprivileged site. Sertoli cells are epithelial cells within the seminiferous tubules and are responsible for the synthesis of a wide variety of proteins and hormones required for the orderly differentiation of sperm cells (*11*). Our hypothesis was that certain of these factors have immunosuppressive qualities that result in the protection of intratesticular islet allografts against immune destruction. In order to test this hypothesis, a series of experiments were initiated, in vitro, to examine the effects of Sertoli cell factors on T lymphocyte proliferation, and on islet cell function. The present report deals with the effects of Sertoli cell secretory products on T lymphocyte proliferation.

MATERIALS AND METHODS

Isolation and incubation of Sertoli cell enriched fractions. Male Sprague-Dawley (S-D) rats, between 16 and 18 days old, were used exclusively, since at this age testicular contamination with sperm cells is less than 5%. The cells were prepared according to a modification of the method of Morrid et al. (*12*), which involved the following: the rats were anesthetized with pentobarbital sodium (Wyeth Laboratory Inc., Philadelphia, PA) and the testes removed under aseptic conditions. The membranes were gently peeled off and the seminiferous tubules cut into smaller (1.0-mm) fragments. The fragments were then digested, in tandem, first with trypsin (type III, Sigma Co., St. Louis, MO), 75 mg/8 testes, and then with a mixture of collagenase (type IA, Sigma, St. Louis, MO), 40 mg/8 testes, and DNAase (Sigma, St. Louis, MO), 20 μg/8 testes, in a shaking water bath at 37°C. The digested material, consisting mainly of Sertoli cells, was washed three times with a phosphate buffer solution. The Sertoli cells were suspended in culture medium, Ham-F_{12}-MEM, supplemented with 1% fetal calf serum, and were transferred to 24-well plates (Costar Co., Cambridge, MA). Each

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well was coated with Matrigel (Collaborative Research Inc., Bedford, MA) and contained 1 ml of the Sertoli cell suspension. The plates were preincubated at 39°C for 2 days in order to eliminate residual germ cells. The supernatants were aspirated and Sertoli cells were then cultured in serum-free Ham-$F_{12\text{-}D}$MEM, supplemented with follicle stimulating hormone (10 µg/dL, NIADDK, NIH, Bethesda, MD), testosterone (3 µg/dl, Sigma, St. Louis, MO), retinal acetate (5 µg/dl, Sigma, St. Louis, MO), and a combination of insulin, transferrin, selenious acid, linoleic acid (ITS$^+$), 1 ml/dl (Collaborative Research, Bedford, MA), at 32°C or at 37°C for a total of 6 days. The conditioned media were collected at daily intervals, centrifuged at 500 ×g to remove the debris, pooled, and stored at −20°C.

Rat spleen lymphocyte preparation. Spleens were removed from male or female S-D rats, weighing between 250 and 300 g, under sterile conditions. Single-cell suspensions were prepared by gentle homogenization of the tissues. The cells were centrifuged at 500 ×g and the pellets resuspended in HBSS and 20% acid citrate dextrose in order to lyse the erythrocytes. The cells were washed in a phosphate buffer solution and centrifuged at 2000 rpm for 10 min. The cells were then counted after being suspended in Ham-$F_{12\text{-}D}$MEM, supplemented with 10% normal rat serum (NRS) and antibiotics.

Lymphocyte proliferation (LP)[a] assay. Spleen lymphocyte preparations were suspended at 2×10^6/ml in Ham-F_12-$_D$MEM containing 10% NRS. A total of 50 µl of the cell suspension was dispensed per well, in triplicate, in 96-well U-bottomed culture plates, and media added to a total volume of 200 µl/well. In studies where different volumes of Sertoli cell enriched media (SSC) were added to the lymphocyte cultures the final volume per well was always brought to 200 µl by addition of the appropriate amount of media. An optimal concentration of 2 µg of Con A was added to each well in order to stimulate lymphocyte proliferation. Culture plates were incubated at 37°C for 5 days in a humidified atmosphere of 5% CO_2 in air. The cultures were pulsed with 1 µCi/well of ^{3}H-thymidine (sp. activity 6.7 µCi/mmol) 18 hr before the cells were harvested in a Titertek harvester. The dried strips were then collected and processed for counting in a liquid scintillation counter. Viability of the cultured spleen cells was assessed, at daily intervals, by means of a 0.2% trypan blue exclusion test.

Temperature-induced effects. Sertoli cells were cultured at 32°C or at 37°C and the spent media (SSC) collected and stored at −20°C. Lymphocytes were prepared as described above. Cultures for proliferation studies were prepared, in triplicate, by adding 25 µl of 4×10^6/ml of the cell suspension, and 2 µg of Con A per well. Varying amounts of SSC, obtained from cultures at 32°C, or 37°C were added to the lymphocyte cultures and the final volume was brought to a total volume of 200 µl/well with Ham-F12-DMEM. Control wells contained lymphocytes, with and without Con A, and with Ham-F12-DMEM but no SSC. The plates were cultured at 37°C for 3 days before harvesting.

Hormone-induced effects. Sertoli cells were cultured at 37°C for 2 days in the presence of (A) Ham-F12-DMEM and FSH, testosterone, retinal acetate, and ITS$^+$, (B) the same as (A) but without FSH, or (C) the same as (A) but without testosterone. Lymphocyte cultures were prepared as described above and cultured in the presence of varying amounts of the crude SSC. The control wells for all of these experiments contained cells and Ham-F12-DMEM supplemented with FSH, testosterone, retinal acetate, and ITS, but no SSC. The culturing and processing of the cells were done as described under (A) above.

Effect of heat-treatment of SSC on lymphocyte proliferation. Sertoli cells were cultured at 37°C for 2 days in Ham-F12-DMEM supplemented with FSH, testosterone, retinal acetate and ITS. The SSC was collected and heated at 56°C in a water bath for 30 min before addition to the lymphocyte cultures.

Effect of timing of addition of SSC on lymphocyte proliferation. Sertoli cells were cultured at 37°C for two days and in the presence of the four additives, as described above. Lymphocyte cultures were prepared, and 50 µl of the SSC added per well at the beginning of the culture or at intervals of 1, 15, 24, 36, and 48 hr later. The cells were then cultured and processed as described above.

Effects of SSC on the production of IL-2 by rat spleen cell cultures. IL-2 dependent CTLL assay: Rat spleen cell suspensions were prepared in Ham-F12-DMEM supplemented with 10% NRS and 5×10^{-4} M 2-mercaptoethanol (Sigma, St. Louis, MO). Control plates were prepared in triplicate by adding 100 µl of a 1×10^6/ml cell suspension and 100 µl of 20 µg/ml Con A per well in 96-well culture plates. Test wells were prepared in triplicate by adding the same number of cells and varying amounts of SSC per well. To each well Con-A–containing media were then added to bring the final volume to 200 µl and the final Con A concentration to 2 µg/ml. The plates were cultured at 37°C for 24 hr. The media were then removed, centrifuged at 2000 rpm for 5 min, and the supernatants collected to test IL-2 content in a bioassay using IL-2-dependent CTLL cells.

The CTLL assay: We used the CTLL mouse cell line known to proliferate in response to IL-2, but not to Con A, as the indicator cell system for determination of IL-2 activity. Supernatant derived from cultures of Con A–stimulated rat spleen lymphocytes in the absence and presence of SSC was used as a source of IL-2. CTLL cells were maintained and cultured in 10% FCS Ham-F12-DMEM media supplemented with 2-ME. To test the effects of SSC on IL-2 production by rat spleen cells, cultures were prepared in triplicate by adding 100 µl of a 1×10^4 CTLL/ml suspension, and 100 µl of the rat spleen cell supernatant fractions per well in U-bottomed 96-well plates. The cells were cultured at 37°C for 24 hr. IL-2 activity was assessed by measuring ^{3}H-thymidine uptake by CTLL after a 6-hr pulse with 1 µCi ^{3}H-thymidine (sp. activity 6.7 µCi/mmol) at the end of the 24-hr period.

Lymphocyte proliferation: After removal of the supernatant for the CTLL assay, the remaining cells were collected, and 100 µl of the mixed cell suspension was resuspended with 100 µl/well of fresh Ham-$F_{12\text{-}D}$MEM in a fresh culture plate. The plates were then cultured for an additional 2 days at 37°C before harvesting.

Effect of SSC on IL-2 responsiveness of rat spleen lymphocytes. In these studies each well containing 50 µl of a lymphocyte cell suspension (2×10^6/ml) in media containing 2 ME, 25 µl of Con A (80 µg/ml), varying amounts of SSC (0, 25 µl, 50 µl, 75 µl, and 100 µl/well), varying amounts of IL-2 (Sigma, St. Louis, MO), and varying amounts of media with 2-ME, were added to a total volume of 200 µl.

Statistics. All data are expressed as means ± SE. Statistical analysis was done with the Student's *t* test, and values were considered significant if *P* was less than 0.05.

RESULTS

Temperature-induced effects on Sertoli cell cultures. Our previous studies demonstrated that immunological privilege of the abdominal testis was superior to the testis in the scrotal position. We reasoned that the difference observed could be attributed to the temperature environment when testes are present in these two positions. Therefore, we asked if temperature affects the production of a suppressor factor(s) by Sertoli cells. The effects of SSC obtained from Sertoli cells cultured at different temperatures on lymphocyte proliferation are shown in Figure 1. SSC obtained from cultures maintained at 37°C inhibited lymphocyte proliferation in a dose-dependent manner. The degree of inhibition with SSC obtained at 37°C was significantly (P<0.05–0.001) greater than the inhibition of SSC obtained from cultures kept at 32°C at each of the three different concentrations tested.

A possible explanation for SSC inhibition of lymphocyte proliferation is that it could be directly toxic to the cells. Serial determinations of cell viability failed to demonstrate a toxic effect at any of the concentrations of SSC used (Fig. 2).

Hormone-dependence of Sertoli cell cultures. To determine if

[a] Abbreviations: SSC, Sertoli cell–enriched medium; LP, Lymphocyte proliferation.

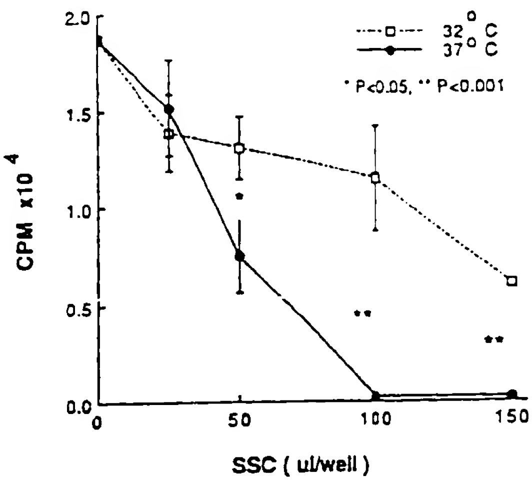

FIGURE 1. Temperature dependence of the production of the inhibitor/s by cultured Sertoli cells. Inhibition of Con A-stimulated spleen lymphocyte proliferation by various concentrations of Sertoli cell-enriched media (SSC) obtained from Sertoli cells cultured at 32°C or at 37°C. The values for ^{3}H-thymidine uptake are plotted as mean ± SEM.

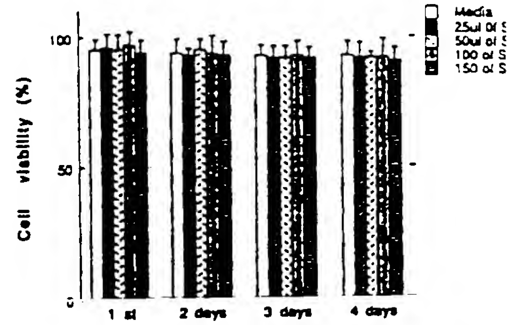

FIGURE 2. Effects of SSC on the viability of lymphocytes. Perc age viability of Con A-stimulated spleen lymphocytes cultured in presence of various concentrations of Sertoli cell-enriched media (S for periods ranging between 1 and 4 days. The values are plotte mean ± SEM.

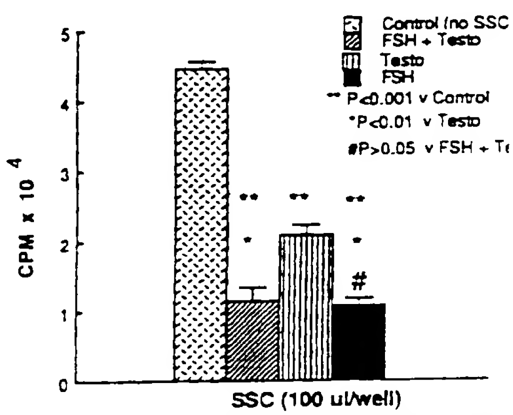

FIGURE 3. Hormone dependence of the production of the inhibi factor/s by Sertoli cells. Con A-stimulated proliferation of sp lymphocytes cultured in the presence of 100 μl/well of Sertoli c enriched media (SSC). The media were harvested from Sertoli c cultured at 37°C in the presence of Ham-F_{12}-DMEM, without addit (control), in the presence of Ham-F_{12}-DMEM supplemented with t FSH and testosterone, in the presence of Ham-F_{12}-DMEM sup mented with only testosterone, and in the presence of Ham-F_{12}-DM supplemented with only FSH. The values are plotted as mean ± SI

the production of the suppressor factor(s) was influenced by testicular hormones, Sertoli cells were cultured in the presence of FSH, testosterone, or a combination of both. As shown in Figure 3, Sertoli cells produced SSC that was more immunosuppressive than cells cultured without these hormones. Cells cultured in the presence of FSH at 37°C, with or without testosterone, showed the greatest immunosuppression.

Effects of preheating of SSC on lymphocyte proliferation. Figure 4 shows that heating SSC to 56°C for 30 min failed to remove the inhibitory effect of any of the SSC tested.

Effects of timing of addition of SSC on lymphocyte proliferation. Figure 5 shows that significant suppression of lymphocyte proliferation occurred if the SSC was added to the cultures within the first 15 hr after initiation of culture. SSC added to the cultures between 24 and 48 hr had no inhibitory effect on lymphocyte proliferation.

Effects of SSC on IL-2 production. Next we asked if the SSC affects IL-2 production or IL-2 responsiveness of T cells, or both. Figure 6 shows that the growth of CTLL cells was inhibited in a dose-dependent manner by the addition of supernatant-containing SSC to rat spleen cells cultured with Con A. The values at each of the SSC doses tested were significantly lower than corresponding values of the control, cultured in the absence of SSC. Lymphocyte proliferation, which was done in a parallel experiment, showed a similar dose-dependent pattern of inhibition by supernatant-containing SSC (Fig. 5). IL-2 added back to the cultures at concentrations of 0.5 U and 1.0 U/well failed to overcome the inhibitory effects of SSC on lymphocyte proliferation (Fig. 7).

DISCUSSION

The basis of the present study was our observation that islet allografts injected into the abdominal testes of nonimmunosuppressed, spontaneously diabetic BB/Wor rats not only lead to a reversal of hyperglycemia within 48 hr but survive ind nitely, in contrast to identical preparations injected into scrotal testes (7). In order to examine the mechanisms testicular immune privilege, the present studies were desig to create in vitro conditions that would mimic those found vivo. It was postulated that Sertoli cells produce a factor factors that have the capacity to inhibit the local immu response—or, conversely, the factors secreted by the Ser cell may, in some way, have altered the immunogenicity of grafted islet cells.

The initial series of experiments demonstrated that Ser cell-enriched media inhibit lectin-induced lymphocyte pro eration in vitro. The single most striking finding of this stu

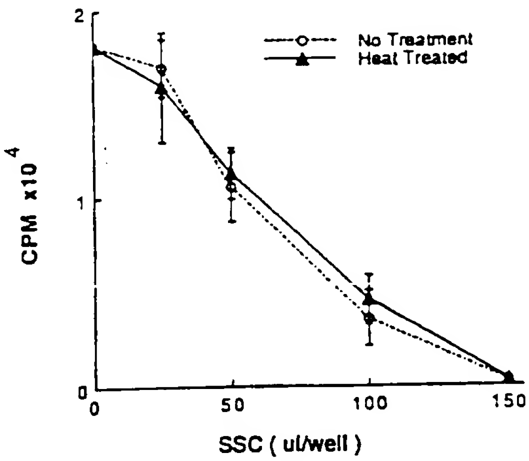

FIGURE 4. Heat stability of the inhibitory factor/s produced by the Sertoli cell cultures. Inhibition of Con A-stimulated spleen lymphocyte proliferation in the presence of various concentrations of Sertoli cell-enriched media (SSC). The SSC had been left untreated or were preheated for 30 min at 56°C prior to addition to the lymphocyte cultures. The values are expressed as mean ± SEM.

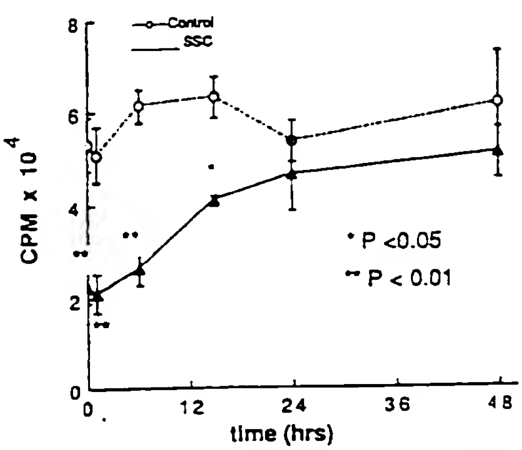

FIGURE 5. Effects of timing of addition of SSC on lymphocyte proliferation. Lymphocyte proliferation in the presence of media alone (broken line) or in the presence of 100 μl/well of SSC (solid line) added at different intervals after the initiation of lymphocyte cultures. The values are expressed as mean ± SEM.

was how temperature-dependent this phenomenon was. Thus media collected from cells maintained at 37°C during culture were significantly more suppressive than media collected from cells cultured at 32°C. Two different mechanisms may have been involved to explain these results—at a higher temperature larger quantities of an inhibitor may have been made, or a different, but more potent, factor was produced at a higher temperature. The experiments done to date cannot distinguish between these two possibilities. It is tempting, however, to

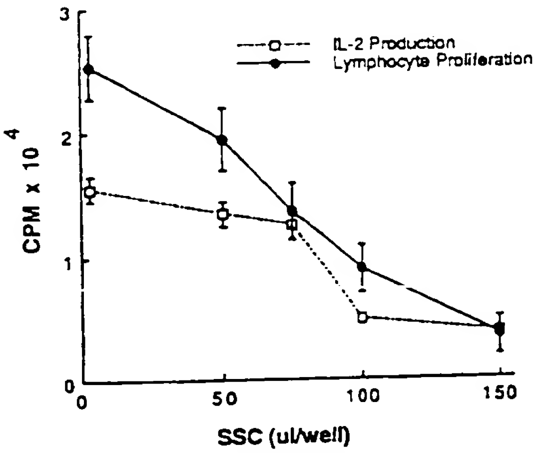

FIGURE 6. Effect of SSC on IL-2 production and proliferation of spleen lymphocytes. Rat spleen lymphocytes were stimulated with 2 μg/ml Con A in the presence of the indicated concentrations of SSC. At 24 hr aliquots of the culture supernatants were removed and assayed for IL-2 activity in a bioassay using CTLL (broken line). IL-2 activity is presented in triplicate as cpm ^{3}H-thymidine uptake by CTLL cells ± SEM. The lymphocyte cultures were allowed to incubate for an additional 48 hr and then pulsed with ^{3}H-thymidine. Proliferation of spleen lymphocytes (solid line), was measured as mean triplicate cpm ^{3}H-thymidine uptake ± SEM.

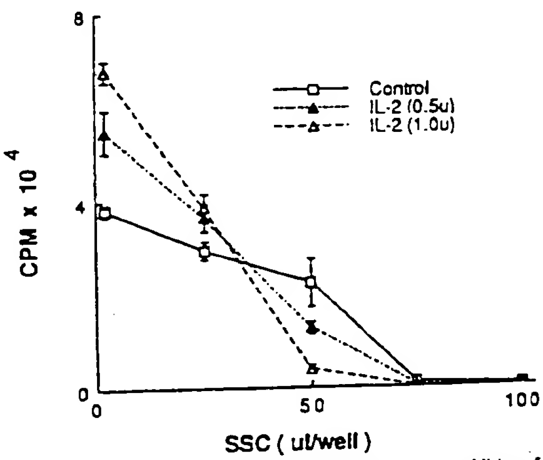

FIGURE 7. Responsiveness of spleen lymphocytes to the addition of exogenous IL-2. Con A stimulated the proliferation of spleen lymphocytes, which were cultured in the presence of Sertoli cell-enriched media (SSC), with no IL-2 added to the cultures (control), or in the presence of IL-2 added at a concentration of 0.5 U/well, or 1.0 U/well. The values are expressed as mean ± SEM.

propose that the inhibitory effects of this factor(s) may be partially responsible for the protection of islet grafts against rejection in vivo at 37°C, and to observe that even presensitized lymphocytes, which are able to penetrate within the testis, are

nonetheless not able to initiate the rejection of islet xenografts (*13*).

Our results showed that the production of this factor by Sertoli cells is hormone-dependent. Of the various reagents tested, both testosterone and FSH apparently enhanced the production of the inhibitor, although FSH was clearly the more potent of the two hormones. FSH at a low concentration stimulated the production of the inhibitor even in the absence of added testosterone. These results also tend to confirm our previous observations made in vivo, which showed that neither the induction nor the maintenance of extended islet allograft survival requires functioning Leydig cells (*10*). In these experiments a toxin that selectively destroys Leydig cells was used prior to the transplantation of diabetic rats. Even though the testicular testosterone content decreased to castrating levels, and ultrastructural studies confirmed the complete absence of Leydig cells, intratesticular islet allografts survived for extended periods in nonimmunosuppressed recipient rats (*10*).

It is documented that FSH affects Sertoli cell function by binding to specific membrane receptors (*14*). Interaction of FSH with Sertoli cells, moreover, is a temperature-dependent phenomenon, with optimal binding occurring at 37°C and a sharp decline occurring at lower temperatures (*14*). As with other protein hormones, FSH binding to Sertoli cell membranes results in an activation of adenyl cyclase and a rapid rise of intracellular cAMP levels (*15*). In this particular context it is of interest that Gonzales et al. (*16*) showed that the production of inhibin by isolated segments of seminiferous tubules of adult rats occurred in a dose-dependent manner after stimulation with FSH. Furthermore, testosterone did not affect basal inhibin production but caused an inhibition of FSH-induced secretion of inhibin at a higher dose of 10^{-4} M. In agreement with this report, Bardin et al. (*12*) demonstrated that the addition of FSH to Sertoli cell cultures in vitro leads to a rapid rise in inhibin levels in the supernatant fractions. Inhibin, either alone or in the presence of other Sertoli cell secretory factors, may be responsible for the inhibition of the local immune response in the testes. Although this is an attractive hypothesis, confirmation will require further investigation.

The mechanisms by which the factor secreted by Sertoli cells inhibit lymphocyte proliferation have not been defined. One possible explanation apparent from our observations is that Sertoli cell–conditioned media inhibited the production of IL-2 in a dose-dependent manner. The addition of exogenous IL-2 to the lymphocyte cultures was not able to reverse this inhibition, indicating that SSC inhibits both IL-2 production and IL-2 responsiveness of T cells. The SSC inhibitor appears to be effective only if added during the first 15 hr of culture initiation. This confirms that the inhibition of lymphocyte proliferation is not due to toxicity of the SSC inhibitor. Studies are now in progress attempting to isolate the immunosuppressive factors secreted by the Sertoli cell, and to further identif their physiological and immunological properties.

Acknowledgments. We thank Karen Whittington and Ning Nin Miao for excellent technical assistance.

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EXHIBIT H

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IMMUNOSUPPRESSIVE MOLECULES PRODUCED BY SERTOLI CELLS CULTURED IN VITRO: BIOLOGICAL EFFECTS ON LYMPHOCYTES

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In the present study we have analyzed the proteins secreted in vitro by murine Sertoli cells to identify immunosuppressive factors. Our data show that Sertoli cells secrete molecules capable to inhibit proliferation of lymphocytes activated in vitro. Cytophluorimetric analysis indicates that treated cells are arrested in the G_1 phase of cell cycle. The inhibitory activity is specific for both B or T lymphocytes but not for other non-lymphoid cells and is associated to proteins, heat and freeze stable, with Mr of more than 30 kDa. Lymphocytes treated with Sertoli immunosuppressive proteins drastically reduce the secretion of interleukin-2. © 1992 Academic Press, Inc.

pd. 14/08/1992 p. 1639-1646 8

The testis has long been known as an immunologically privileged site of the body. Germ cells expressing auto-antigens are present in the seminiferous tubules since puberty (1-5), but immune-competent interstitial cells do not show any sign of activation; moreover interstitial tissue can tolerate for long periods of time allo-transplants (6-8). Several hypotheses have been proposed to explain such peculiar behavior of the immune system in the testis. Among these are: the low testicular temperature, the presence of immunosuppressive steroids and the absence of immuno-competent cells. All these hypotheses, based on morpho-functional characteristics of the testis, have been considered either inconsistent or not adequate to explain the phenomenon (7, 9-12). As for the blood-testis barrier, which was supposed to segregate germ cells expressing auto-antigens, it has been shown to be incomplete in some areas (13) and the presence of autoantigens has been also demonstrated "outside" the barrier itself (14-15). Attention has therefore been directed to the identification of soluble factors

Abbreviations: SIP, sertoli immunosuppressive proteins; TGF-β, transforming growth factor-β; IL-2, interleukin-2; PBS, phosphate-buffered saline; FSH, follicle stimulating hormone; PMA, phorbol 12-myristate 13-acetate.

0006-291X/92 $4.00

produced locally and having the function to prevent immune reactions. In the last few years some preliminary attempts have been made to achieve informations on the presence of immunosuppressive activity in testicular fluids or in Sertoli cell culture media (16-18). The purpose of the present paper is to gain further insights into the nature and the functional characteristics of immunosuppressive factor(s) produced by Sertoli cells.

MATERIALS AND METHODS

Sertoli cell cultures: Sertoli cells were prepared from 18-day old CD1 mice by sequential digestion with trypsin and collagenase (Boehringer Mannheim, Germany) as previously described (19). Cells were cultured at 32° C 95% air and 5% CO_2 in serum free MEM (Gibco, USA).

Sertoli cell protein preparation: Sertoli cells conditioned media were collected after 5 days of culture, concentrated and desalted by Centriprep ultrafiltration devices (Amicon, Danvers, Mass). The samples were first fractionated on Centriprep 30; the retentate containing molecules with molecular weight above 30 kDa was collected, while the eluate was further fractionated on Centriprep 10 to obtain the fraction with molecules ranging from 10 to 30 kDa. Both fractions were divided into aliquots and lyophilized. Protein content of the aliquots was determined by using the micro BCA assay (Pierce, Rockford).

Preparation of splenic T lymphocytes: Spleens from adult CD1 mice were aseptically removed and gently dissociated in culture medium. Red blood cells were removed by treatment with ACK-lysing buffer (GIBCO) and T cell isolated by incubation of the cell suspension on nylon wool column (Polyscience, PA, USA) (20). T Lymphocytes, resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine 50 μM 2-mercaptoethanol and 50 μg/ml gentamicin, were stimulated to proliferate with anti-mouse CD3 mAb (21), with lipopolysaccharide (LPS) 10 μg/ml or with PMA 10 nM and 0.3 μM ionophore A23187 (Sigma St. Louis, MO) with or without SIP, in four replicate wells. Microcultures (2×10^5 cells/well) were set up in 96 well plates (Costar, Cambridge MA), incubated for 72 h at 37° C in 95% air and 5% CO_2 and pulsed for the last 12 h with [^{3}H] TdR (0.5μCi/well, A.S 6.7 Ci/mmol) (NEN Dupont, GmbH). Cells were then harvested onto fiber glass filters and incorporated radioactivity measured.

Assay of IL-2 activity: Supernatants from activated T lymphocytes cultivated with or without SIP were collected at different times and assayed for IL-2 activity by their ability to support the growth of the murine IL-2 dependent CT4R cell line (22).

Antibody neutralization of TGF-β_1 activity: For identification of immunosuppressive activities related to TGF-β_1 SIP (4 μg/ml) was preincubated for 2 h with neutralizing antibodies to TGF-β_1 (3μl/well provided by Dr. Anita Roberts). The specificity of the antisera was confirmed by using purified TGF-β_1 (0.5-2.5 ng/ml).

Cytometric analysis: Cells from replicate wells were pooled, washed and fixed (methanol-acetone 4:1 v/v at 50% in PBS at 10^6 cells/sample. Forward light scatter for volume and red fluorescence (>610 nm) of propidium iodide (50μg/ml in PBS with 100 KU/ml RNAase A) for DNA content analysis were measured by an EPICS 541 (Coulter, Hialeah, Fla.) flow cytometer.

RESULTS

T-lymphocytes, induced to proliferate by monoclonal antibodies to CD3 were challenged with proteins secreted by Sertoli cell cultures. As shown in Fig. 1, the fraction including Sertoli proteins with molecular weights above 30 kDa produces a marked

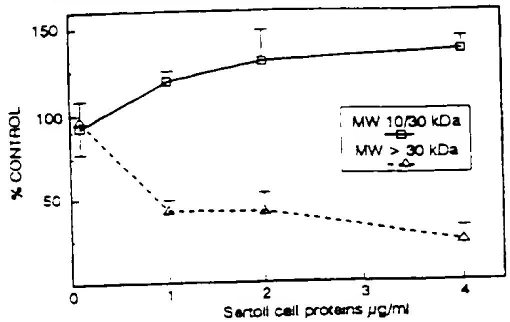


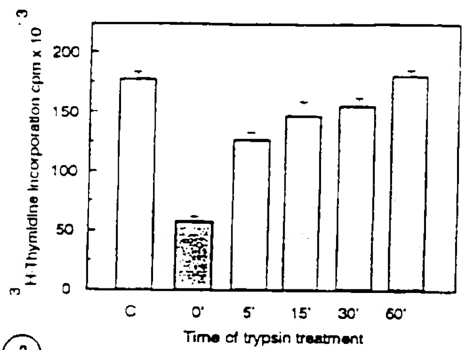
Fig. 1. ^{3}H-Tdr incorporation by splenocytes stimulated with anti-CD3 mAb in the presence of two molecular fractions of Sertoli cell secreted proteins. The fraction > 30 kDa (SIP) shows immunosuppressive activity. Each point represents the average of quadruplicate samples.

inhibition of the proliferative activity of lymphocytes, while the fraction including molecules with M.W. between 10 and 30 kDa does not inhibit lymphocyte proliferation. Same results were obtained when the immunosuppressive fraction (SIP) is added to B-lymphocytes stimulated to proliferate with lipopolysaccaride (Tab. 1). When tested on lymphoid and non lymphoid cells, SIP do not inhibit the proliferative rate of the latter as measured by ^{3}H-thymidine incorporation; instead, they potentiate the effect of serum on the growth of these cells (Tab. 1). Such lymphocyte specific anti-blastic activity is associated to proteins, since treatment of SIP with trypsin completely abolished their

TABLE 1

[^{3}H]TdR incorporation by different cell types treated with SIP (4µg/ml)

CELL TYPES	% OF CONTROL (± SEM)
PRIMARY CULTURES	
- B LYMPHOCYTES	27 (± 4)
- T LYMPHOCYTES	25 (± 6)
- TESTICULAR FIBROBLASTS	100 (± 5)
CELL LINES	
- CT4R (IL2-dependent)	98 (± 3)
- CT4S (IL-4 dependent)	50 (± 5)
- B9 (IL6-dependent)	65 (± 2)
- NS-1 MYELOMA	88 (± 4)
- NIH-3T3 FIBROBLASTS	150 (± 7)



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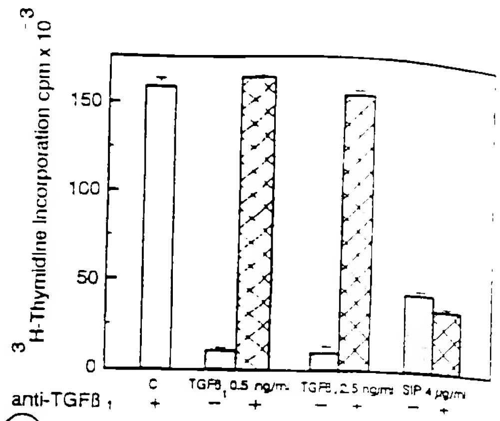

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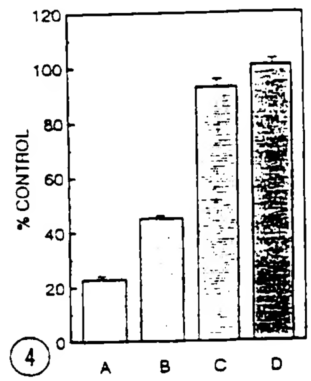
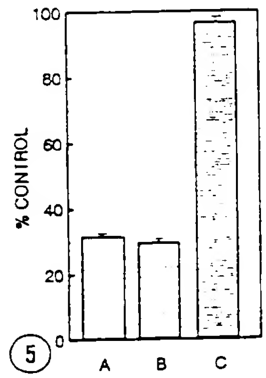
Fig. 2. Effect of trypsin treatment of SIP. SIP (4 μg/ml) and control medium were incubated with 0.025% trypsin for the indicated times. The samples were then assayed for their ability to inhibit the proliferation of T lymphocytes. Each point represents the average of quadruplicate samples.

Fig. 3. Effect of anti-TGF-β_1 neutralizing antibodies on the proliferation of splenocytes. SIP, control medium, and purified TGF-β_1 were incubated for 2 h with (+) or without (-) anti-TGF-β_1 neutralizing antibodies before addition to the T lymphocytes in the proliferation assay.

biological effects on T and B-lymphocytes (fig. 2). SIP retain their biological activity after treatment at 56° C for 30' or at 100° C for 5' (not shown) as well as after repeated freeze thawing or lyophilization (see Material and Methods).

TGF-β_1 is known to be an immunosuppressive agent capable of inhibiting lymphocyte proliferation (23), and evidence has been published indicating its production by Sertoli cells (24,25). As shown in figure 3, addition of TGF-β_1, to stimulated T-lymphocyte, completely inhibits their proliferation and such effect can be fully reverted by the addition of neutralizing anti-TGF-β_1 antibodies. Anti-TGF-β_1 neutralizing antibodies however, are not able to revert the effect of SIP on lymphocyte proliferation (Fig. 3). Pre-incubation of both lymphocytes or anti-CD3 coated culture plates with SIP, followed by washings, did not affect the normal proliferative pattern of the cells (fig. 4, C-D). Addition of SIP after 3 hrs from the beginning of anti-CD3 stimulation did indeed produce inhibition of proliferation as well (fig. 4 B). Experiments have been performed in which the proliferative stimulus to lymphocytes did not come from surface ligands but from a simultaneous direct intracellular activation of PKC by PMA and an increase of Ca^{++} levels by ionophore A23187 (26). Under these conditions SIP retain their ability to inhibit lymphocyte proliferation (fig. 5 A) also when added 3 hrs after the stimulus (fig. 5 B). Pre-incubation of the cells with SIP does not produce any effect. The DNA content and the volume of the cells have been evaluated by cytometric analysis. As seen in Table 2, the treatment of activated lymphocytes with SIP produces a dramatic reduction in the number of cells entering the S phase of the cell cycle and an increase of those in G_1





Figs. 4 and 5. ^{3}H-Tdr incorporation by splenocytes stimulated with immobilized anti-CD3 mAb (Fig.4) or PMA-ionophore (Fig.5). Cells have been treated as follows: **A)** stimulus and SIP from time 0 h; **B)** stimulus from time 0 h and stimulus + SIP from time 3 h; **C)** SIP from time 0 h, washings and stimulus from time 3h; **D)** from time 0 h with anti-CD3 mAb previously incubated for 3 h with SIP which has been removed by washings.

Both treated and control cells increase their size during the first 44 hr of incubation whereas after that time only control cells continue to grow (Tab. 2). Production of IL-2 by control or SIP-treated T-lymphocytes has also been evaluated by a bioassay using an IL-2 dependent cell line. The results shown in fig. 6 indicate that the production of IL-2 is inhibited by SIP as early as 17 hours after the beginning of the treatment.

DISCUSSION

Our data demonstrate that Sertoli cells cultured in vitro secrete protein(s) (SIP) which specifically inhibit the proliferation in vitro of either T or B lymphocytes but not of

TABLE 2

Cytometric analysis of DNA content and volume of splenocytes in control condition (C) or under treatment with SIP 4 μg/ml (T)

TIME (h)	VOLUME (a.u)		G_1 (%)		S (%)		G_2 / M (%)	
	C	*T*	C	*T*	C	*T*	C	*T*
0	74		95		4		1	
18	81	***85***	91	***94***	6	***5***	3	***1***
44	104	***97***	60	***90***	35	***9***	5	***1***
52	130	***105***	50	***81***	43	***18***	7	***1***
70	123	***106***	56	***80***	39	***18***	5	***2***

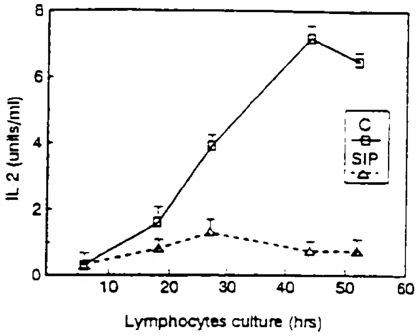


Fig. 6. Kinetics of IL-2 production by lymphocytes. T lymphocytes stimulated with anti-CD3 mAb were incubated with (---) or without (___) 4μg/ml of SIP and supernatants were collected at the indicate time intervals. The supernatants were diluted 1/3 and assayed for their ability to stimulate CT4R proliferation as determined by [^{3}H]TdR incorporation. Units of IL-2 are based on comparison with a standard curve with purified IL-2. Each point represents the average of quadruplicate samples.

other non-lymphoid cell types tested. This inhibition is probably mediated by a suppression of IL-2 secretion.

These data are in partial agreement with those of Wyatt et al. (17) who, following some our preliminary report on the same issue (16), have described the presence of an anti-lymphocytic activity in Sertoli cell conditioned media. These authors however reported a presuntive molecular weight of 10-25 kDa for their factor(s) which suppress proliferation of various lymphoid and non lymphoid cell types at concentrations of proteins about ten times greater than those used in the present work.

We have found that, during the process of lymphocyte activation, SIP do not act at the level of ligand\receptor interactions nor by altering the transduction of the membrane signal. SIP, in fact, maintain their activity when pre-incubated with the anti-CD3 mAb or with the surface of the cells and when administered to the cells 3 hrs after the beginning of surface stimulation with anti-CD3 mAb. Moreover, SIP inhibit also the proliferation of lymphocytes stimulated with PMA and ionophore, when the ligand/receptor binding on the cell surface and the regulation of membrane signal transduction have been by-passed.

After antigenic activation T-cells undergo morphologic changes (blastogenesis) that preceed the entrance in the S phase and cell division. The increase in cellular size during the first period of stimulation with anti-CD3 mab does not show any significant modification when cells are treated with SIP. This observation suggests that the enlargement of the cells, representing an early morphologic sign of commitment to proliferation (27), is not affected by treatment with SIP.

Following antigen stimulation, one of the fundamental steps in the activation of lymphocytes is the expression of the gene for IL-2. The transition from G_1 into the replicative phases is mediated by IL-2 through an autocrine mechanism (28). The receptor for IL-2 is composed of two subunits, α and $-\beta$, which can independently bind

IL-2 with low and intermediate affinity, respectively (29). Thus, whereas resting T cells express β-subunit and are able to respond to very high doses of IL-2, antigenic stimulation results in the induction of IL-2 secretion which in turn activates the expression of the α-subunit and the formation of the high affinity α/β-IL-2R, enabling activated T cells to respond to very low concentrations of IL-2. We have demonstrated that lymphocytes treated with SIP show, respect to the control, a decreased secretion of IL-2 in the medium. The anti-proliferative activity of SIP therefore seems to act through an inhibition of the mechanisms that activate the synthesis of IL-2. Since progression from G_1 to S phase is mediated by IL-2, inhibition of IL-2 secretion by SIP induces arrest in G_1, as shown by cytometric evaluation of DNA content of the cells. These biological effects of SIP on target cells show striking similarities with those of known immunosuppressive drugs such as Cyclosporin A and FK506 (30). TGF-β_1, a well known immunosuppressive factor (23), is produced by Sertoli cells (24) and its production is down regulated by FSH (25). SIP obtained from Sertoli cells treated with FSH, do not show any difference in their inhibitory effect on lymphocyte proliferation (not shown). This result indicate that the possible contribution of TGF-β_1 to the immunosuppressive activity of SIP is not relevant. Moreover, experiments performed with specific anti TGF-β_1 neutralizing antibodies show that the immunosuppressive activity present in SIP cannot be referred to proteins related to TGF-β_1. We therefore propose that Sertoli cells can contribute to the maintenance of the immunological privilege in the testis by secreting in the surrounding environment specific anti-lymphoblastic protein(s). Further investigations on the nature of these molecules require a biochemical characterization of SIP that is presently in progress.

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EXHIBIT I

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Original Contribution

SERTOLI CELL-ENRICHED FRACTIONS IN SUCCESSFUL ISLET CELL TRANSPLANTATION

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☐ **Abstract – Prolonged survival of Islet- allo- and xenografts can be induced following implantation of the islets into the abdominal testis of diabetic rats. We previously showed that a factor released by Sertoli cells appears to be responsible for the protection of the intratesticular islet allo- and xenografts against rejection. The aim of this study was to examine whether an immunologically privileged site can be established in an organ site in vivo, other than the testis, such as the renal, subcapsular space, to make feasible the grafting of female recipients as well. A total of 36 male and 21 female, diabetic, PVG rats were divided into six different treatment groups: 1) Six male rats were grafted with islets from Sprague-Dawley (S-D) donor rats only. 2) Ten male rats were grafted with islets from (S-D) donors and were then given a short course of cyclosporine (CsA) posttransplantation. 3) Ten male rats were grafted with islets from (S-D) donors and with Sertoli cell-enriched fractions (SEF) from PVG donors but without CsA. 4) Ten male rats were grafted with a combination of islets from (S-D) and SEF from (PVG), donors, respectively, and CsA. 5) Ten female rats were given an identical combination of cells and CsA as depicted for group 5. 6) Ten female rats were grafted with a combination of islets and SEF, both cell types from S-D donors, and CsA. The results showed that 70% to 100% of the grafted rats in groups 1, 2, and 3 remained hyperglycemic. Prolonged normoglycemia in excess of 100 days was induced in more than 75% of the grafted rats only in groups 4, 5, and 6, or in those animals who were grafted with a combination of islets and SEF and who were given a short course of CsA as well. Electron microscopic examination of the grafted tissues showed the presence of intact beta cells and of cells with features characteristic of Sertoli cells. Our results suggest that 1) the protection of islet allografts in nonimmunologically privileged site can be achieved in male and female rats by means of the simultaneous transplantation of Sertoli cells. 2) Sertoli cells apparently maintain the capacity to secrete an immune inhibitor in organ sites other than the testis. We conclude that it is feasible to create an immunologically privileged site for the transplantation of isolated islets in male and female diabetic recipients without the need for sustained immunosuppression.**

☐ **Keywords – Sertoli; Allograft; Immunologically privileged.**

INTRODUCTION

Several immunologically privileged sites in mammals sometimes allows prolonged survival of transplanted allografts (1). The immunologically protective mechanisms of the brain and anterior chamber of the eye seem to involve primarily deficient lymphatic drainage (2). Indeed, in some instances, experimental interruption of lymphatic drainage in tissue has created an immunologically privileged site (3). In contrast, the testis (also a privileged site), has excellent lymphatic drainage (4). It is likely that locally produced factors are responsible for inhibition of the immune response (5).

We previously showed that extended survival of islet allo- and xenografts can be achieved after transplantation of isolated pancreatic islet cells into the abdominal testis (6–8). The donor origin of these isolated cells does not seem to influence their long-term survival. Islet cells grafted against major histocompatibility (MHC) barriers (6), islet xenografts (7), and islets of MHC-compatible donors grafted into the testes of rats with autoimmune, spontaneous diabetes mellitus function indefinitely in diabetic recipients (8). However, despite remarkable preservation of function in abdominal, intratesticular islet allografts, there are drawbacks associated with the use of an unconventional organ site. A major concern is that the germ cells may undergo a malignant transformation at the higher core body temperature (9). More importantly, because

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only males have Sertoli cells, this transplantation approach cannot be used in that half of the population which is female.

Because of these objections, and because of the observations that cultured Sertoli cells produce a factor which inhibits the production of IL-2 in vitro (10), studies were initiated in an attempt to create an immunologically privileged site in a heterotopic site, in vivo, other than the testis. In the present study we investigated the effect of Sertoli cell-enriched fractions (SEF) transplanted in conjunction with isolated islets on islet allograft survival in the renal subcapsular space of diabetic rats.

MATERIALS AND METHODS

Animals

PVG rats, weighing between 150-200 g, were used exclusively as recipients of islets. Diabetes was induced by means of a single i.v. injection of 65 mg/dL of streptozotocin. Only rats with plasma glucose levels in excess of 400 mg/dL were transplanted. Sprague-Dawley (S-D) outbred rats were used as islet donors. Either PVG or S-D male rats between 16 and 18 days old were used as Sertoli cell donors.

Islet Preparation

Islets were prepared according to modification of the method of London et al. (11), described in detail elsewhere (6). The islets were purified on Ficoll gradients, and the isolated cells were then incubated for 4 days at 37°C in a humidified atmosphere of 5% CO_2 and air prior to use (6). No special efforts were made to deplete the islets of contaminating passenger leukocytes.

Sertoli Cell-enriched Fraction Preparation

Highly purified preparations of Sertoli cells were isolated from the testes of young males according to the method of Cheng et al. (12). The testes were removed, chopped into several pieces, and placed in a 50 mL conical tube containing 50 mL of Ham's F12/DMEM media. The pieces were washed once by centrifugation at 800 × *g* for 2 min. The supernatant was aspirated, and the tissue resuspended in 40 mL of media containing 40 mg trypsin and 0.8 mg DNase in a sterile 250 mL Erlenmeyer flask. The flask was placed in an 37°C oscillating incubator at 60–90 osc/min for 30 min. This step removed Leydig cells. The tubules were then transferred to a 50 mL conical tube, and centrifuged at 800 × *g* for 2 min. The supernatant fraction was aspirated, and the pellet resuspended in 40 mL of 1 M glycine, 2 mM EDTA containing 0.01% soy bean trypsin inhibitor and 0.8 mg DNase, and incubated at room temperature for 10 min. This step lysed any residual Leydig cells. The cells were washed by centrifugation for 2 min, and the step repeated twice, or until the media was no longer cloudy. The pellet was resuspended by gentle homogenization with a glass Pasteur pipet in 40 mL of media containing 20 mg collagenase in an Erlenmeyer flask, and incubated at 37°C for 5 min with 60–90 osc/min. The cell suspension was centrifuged at 800 × *g* for two min, and the pellet resuspended by gentle homogenization with a Pasteur pipet in 40 mL media containing 40 mg collagenase and 0.2 mg DNase, and incubated in an Erlenmeyer flask at 37°C for 30 min with 60–90 osc/min. The cells were then washed by centrifugation for 2 min, and the process repeated at least three times to eliminate peritubular cells. The cells were resuspended by gentle homogenization with a Pasteur pipet in 40 mL media containing 40 mg hyaluronidase and 0.2 mg of DNase, and incubated at 37°C for 30 min with 60–90 osc/min. The cells were pelleted by soft centrifugation for 2 min, and washed at least five times to eliminate germ cells. The resultant SEF was resuspended in 0.25 mL of media, and immediately transplanted into the recipient rat. Each grafted rat received the equivalent of the total amount of Sertoli cells contained in a single testis.

Transplantation of Rats

The diabetic rat was anesthetized with methoxyflurane USP in a sterile hood and the left flank opened to expose the kidney. The SEF was injected first underneath the renal capsule. The cells could be seen as a milkish bubble underneath the capsule. Immediately afterwards, a total of 10 islets/g of body weight was injected into the same milkish bubble. The needle was retracted slowly to prevent leakage of the grafted cells. Cyclosporine (CsA) was administered s.c. in varying doses over a 20-day period to groups two and four. Because the grafted rats responded similarly whether the drug was administered over a 20-day, or over a 3-day period, all of the subsequent groups, including the female rats, were treated with only three injections of 25 mg/kg CsA, given on days 0, +1, and +2, relative to the graft. The rats received no other therapy.

A total of 36 male and 21 female PVG rats were divided into six different treatment groups: *Group 1*, the control group, consisted of 6 male rats grafted with only islets from S-D donor rats. They received neither SEF nor CsA. *Group 2* consisted of 10 rats grafted with a combination of islets from S-D rats and CsA posttransplantation, but no SEF. *Group 3* consisted of a total of 10 rats grafted with a combination of islets from S-D and SEF from PVG donor rats, but no CsA posttransplantation. *Group 4* consisted of 10 rats grafted with a combination of islets from S-D donors, SEF from PVG donors, and CsA posttransplantation. *Group 5* consisted of 11 female rats grafted with the same combination of cells as depicted for Group four. *Group 6* consisted of 10 female rats grafted with a

combination of islets and SEF, both cell types from S-D donors, and CsA posttransplantation.

Posttransplantation Evaluation of Rats

The grafted rats were transferred to metabolic cages, and plasma glucose levels were obtained at weekly intervals. Urine volumes and urine glucose contents were obtained at daily intervals. A rat was considered cured of the diabetic process if the following criteria were met: A random plasma glucose level $\leq$ 150 mg/DL; aglycosuria; and immediate reversal to hyperglycemia following surgical removal of the grafted kidney.

To determine if any of the rats had become unresponsive to their grafts, normoglycemic rats were challenged with a secondary islet allograft consisting of at least 500, freshly prepared, Sprague-Dawley islets which were injected into the contralateral renal subcapsular space. No immunosuppression was given following the challenge.

To examine the impact of the transplantation of SEF on fertility of the female rats, normoglycemic animals of longer than 30 days were mated with PVG males. Metabolic parameters, as outlined above, were closely monitored, as was the course of their pregnancies.

Structural Analysis of Grafted Tissue

A total of five successfully grafted rats were nephrectomized at intervals following transplantation. Wedge sections of renal tissue, obtained from sites at which islets and SEF had been injected, were prepared for examination by light and electron microscopy, as previously described (15). Briefly, the tissue wedges were immersion-fixed with 5% glutaraldehyde in 0.1 M s-Collidine buffer for 1 h, washed in buffer, and postfixed for 1 h with 1% osmium tetroxide in 0.1 M buffer. Small tissue blocks were cut from the wedges, and dehydrated through a graded series of ethyl alcohols, transferred to propylene oxide, and embedded in Epon 812/Araldite plastic resin. Thick (0.5 μm) and thin (900 ng) sections were stained routinely with toluidine blue and uranil acetate/lead citrate, respectively, for structural analysis by light and electron microscopy.

RESULTS

The results are summarized in Table 1:

Group 1: None of the six rats grafted with islets alone, without either SEF or CsA, became normoglycemic.

Group 2: Three of 10 rats grafted with islets and treated with CsA became normoglycemic for more than 100 days. The 3 normoglycemic rats were challenged with a secondary graft on days 116, 192, and 197, respectively. One rat reverted to hyperglycemia on day 130, while 2 remained normoglycemic.

Group 3: Initially 6 of the 10 rats grafted with islets and SEF, but no CsA, became normoglycemic, but all of them reverted to hyperglycemia by day 14.

Group 4: All 10 of rats grafted with a combination of SEF and islets, and also given CsA became normoglycemic. Two reverted spontaneously to diabetes on days 19 and 76, respectively. Three were nephrectomized on days 58, 84, and 167 following transplantation. All 3 of these rats became hyperglycemic within the next 24 h. The remaining 5 rats were challenged with a secondary islet allograft on days 119, 129, 280, 342, and 400, respectively. Of these, the first 2 reverted to diabetes on day 127 and 139, respectively, while the latter 3 remained normoglycemic.

Group 5: All 11 of the female rats grafted with a combination of islets and SEF, and then given CsA, became normoglycemic. Of these, 4 reverted spontaneously to hyperglycemia by day 28. Of the 7 normoglycemic rats who were mated with male PVG rats, 6 became pregnant, and of these, 8 had litters varying between 1 and 10 pups. They were able to nurse the pups successfully. A total of 7 of the long-term surviving females were challenged with secondary islet allografts at least 200 days following transplantation. None of them reverted to hyperglycemia.

Table 1. Effect of Sertoli cells on islet allograft survival in the non-immunologically privileged renal, subcapsular site

Group (*n*)	Gender	Sertoli cell (donor origin)	CsA	Duration of normoglycemia (days) Individual responses
1 (6)	male	–	–	0,0,0,0,0,0
2 (10)	male	–	+	0,0,0,0,0,0,0,130,>441,>445
3 (10)	male	+(PVG)	–	0,0,0,0,9,10,12,13,13,14
4 (10)	male	+(PVG)	+	19,76,58*,84*,167*,127†,139†,>418†,>422†,>425†
5 (11)	female	+(PVG)	+	7,11,14,28,>287†,>305†,>306†,>308†,>441†,>447†,>457†
6 (10)	female	+(S-D)	+	8,10,96*,128*,>168,>172,>184,>193,>193,>196

*nephrectomized, †challenged with a secondary islet allograft.

Group 6: Of the 10 rats grafted with islets and SEF from the same donor strain of rat, all 10 became normoglycemic. Two reverted to hyperglycemia by day 10. A nephrectomy to remove the graft was done on 2 of the long-term surviving rats on days 96 and 201, respectively. Both reverted to hyperglycemic immediately within the next 24 h.

Tissue Morphology

Renal tissue obtained from the long-term grafted kidney appeared structurally normal by light microscopy (Fig. 1). Transplanted islets in this organ were immediately subjacent to the kidney capsule, and also appeared structurally normal. They displayed tissue and cellular architecture identical to islets in situ (Fig. 1). Individual islet cells were partitioned into cell clusters by thin connective septa containing small vessels and capillaries (Fig. 1). It appeared that most of the islet cells contained secretion granules. When resolved by electron microscopy, islet cells were identified as the β-cell type by the inclusion of ultrastructurally distinctive, and unique insulin-containing secretion granules (Fig. 2). All β-cell clusters observed were in close proximity to intra-islet capillaries (Fig. 2).

There was a high density of cells between, and directly adjacent to, the transplanted islets and renal parenchyma. By light microscopy, they did not appear to be islet cells, kidney cells, nor cells of blood origin (Fig. 1). When observed by electron microscopy, these cells were similar in ultrastructure to Sertoli cells in that their nuclei were irregular in profile, and contained deep nuclear clefts, distinctive nucleoli were often present, and mitochondrial structure was dense. Although these cells did not retain the typical polarity of Sertoli cells in vivo, they were, however, identical in appearance to Sertoli cells in vitro, when the cells are not plated on a basement membrane substrate (33,34). The cells were not associated with a basement membrane, and appeared randomly organized (Fig. 3). Cells showing ultrastructural features of either germ or Leydig cells were not observed.

Fig. 1. Normal pancreatic islets of Langerhans (IL), along with isolated rat Sertoli cells, were transplanted into the subcapsular space in the kidney of diabetic rats. As illustrated in this light micrograph, islets appear structurally normal, and are well-vascularized as indicated by the numerous intra-islet capillaries. The kidney parenchyma (K) also appears normal. Between the islet and renal tissue there is a high density of cells (S) which do not appear to be of islet, renal, or blood origin. Tissue was stained with toluidine blue. ×430.

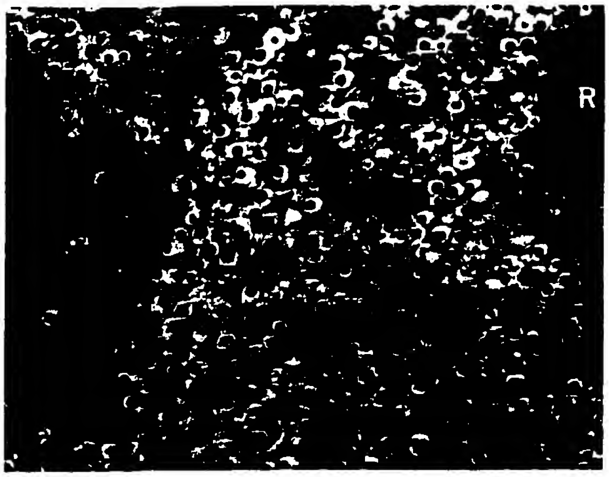

Fig. 2. This electron micrograph shows, at higher magnification, an individual cell within the transplanted islet. Note its close proximity to an intra-islet capillary (R = red blood cell). The unique ultrastructure of its numerous insulin secretion granules (arrows) clearly identifies this cell type as a β-cell. ×6,446.

DISCUSSION

The data reported here show that isolated pancreatic islets, transplanted simultaneously with SEF into the renal subcapsular space of MHC-incompatible donor rats, leads to protection of the islets against immunological destruction. Moreover, in the presence of a minimal amount of CsA, the data show that the rats are capable of developing a state of unresponsiveness or tolerance to their grafted islets that lasts indefinitely.

Fig. 3. This electron micrograph shows, at higher magnification, the fine structure of the extra-islet cells labeled "S" in Fig. 1. Both the cell nucleus (N) and cytoplasm are similar in appearance to Sertoli cells in vivo, and are structurally identical to Sertoli cells in vitro. R = red blood cell. ×3,280.

A number of mechanisms could be envisioned for the prolonged survival of islet allografts under these circumstances. First, despite the fact that special precautions were taken to transplant preparations consisting solely of Sertoli cells, these cells may, nonetheless, have been contaminated with either germ, or with Leydig cells. It has been demonstrated that sperm and seminal fluid suppress phytolectin-induced cell-mediated responses (13). Likewise, others have shown that Leydig cells nonspecifically inhibit lymphoproliferation, in vitro (14). We consider this an unlikely explanation, however, since our earlier studies have demonstrated that the destruction of either germ and/or Leydig cells in vivo does not prevent the long-term survival of intratesticular islet allografts (15), and because no such cell types were observed in the present study. Likewise, testicular steroidogenesis was shown not to be a prerequisite for the protection of intratesticular islet allografts against rejection (16).

Results suggest, rather, that the presence of the grafted Sertoli cells, per se, may have had an impact on the local immune response. Sertoli cells are responsible for the synthesis of many different protein substances, some of which promote growth (17,18), and others which have immunosuppressive capabilities (19). We have previously shown that cultured Sertoli cells produce a factor which inhibits Con-A stimulated lymphocyte proliferation in a dose-dependent manner (10). In this earlier study, lymphocyte proliferation was suppressed, and associated with an inhibition of the lymphokine interleukin-2 (IL-2). It is widely acknowledged that all proliferating T-cells express IL-2 receptors, while resting cells do not, and that interaction of IL-2 with its receptor is an absolute requirement for the clonal expansion of activated T-cells (20). Because the prevention of IL-2 receptor interaction completely inhibits T-cell proliferation, we propose that both clonal expansion and viability of activated T-cells are suppressed by an immuno-suppressive Sertoli cell secretory product (20).

If our premise is correct, then the most puzzling finding of the present study is the observation that transplantation of islets with SEF alone was not sufficient to protect the grafts against immunologic destruction. For successful transplantation, the grafted rats required an additional short course of CsA therapy. In effect, CsA proved to be a much more potent protector of the islets against rejection than did the SEF. For instance, in the control Group grafted with islets alone, but who were given CsA, graft survival occurred in 30% of the recipients (Table 1, Group 2). On

the other hand, in rats transplanted with islets and SEF alone, but without CsA, the grafts did not do nearly as well (Table 1, Group 3). In rats receiving both SEF and CsA, however, the cure rate was quite high, and about 70% of the grafted rats became normoglycemic for longer than 200 days (Table 1, Groups 4–6). We conclude from these findings that the simultaneous presence of SEF and CsA exerted synergistic effects essential for long-term islet allograft survival in the rat kidney.

At present we can only speculate on the mechanism(s) of this apparent synergistic effect. It is conceivable that the presence of SEF, or a factor produced by these cells, protected the islets against the toxic effects of CsA. It has been suggested that CsA may be directly toxic to β-cells (21,22). We regard this as an unlikely explanation. Results of a previous study have showed that β-cell function is not adversely affected in rats transplanted with islet xenografts which were treated with CsA for 100 days (7). Furthermore, in the present study the majority of rats were treated with only three injections of CsA, and this dose is unlikely to have been damaging to the islets.

A more likely explanation is that an immunosuppressive SEF secretory product, or products (20), enhanced, or augmented, the effects exerted by CsA on the immune response. CsA therapy is known to have a major impact on the immune response by means of several different mechanisms. First of all, CsA-induced effects result in a marked suppression of the production of the lymphokine, IL-2, and subsequently, a destruction of specific clones of proliferating cytotoxic T-cells (23-26). This effect would be similar to that caused by the proposed immunosuppressive Sertoli cell factor, as indicated in our earlier study (20). CsA has other modes of action, however, which are quite distinct from that mediated by the Sertoli cell. For example, CsA does not modify IL-2 receptor activity or expression, whereas the immunosuppressive Sertoli cell factor acts like a fusion protein to inhibit IL-2 receptor activity (20). More importantly, however, the treatment of grafted animals with CsA often leads to the induction of a state of specific tolerance or unresponsiveness to allografts (27-30).

Based on the results of this and our previous studies, we can now propose a likely mechanism for the synergistic effects of CsA and Sertoli cells on islet allograft survival: In the presence of both immunosuppressive components, there is a marked depletion of IL-2 production and blockage of IL-2 receptor activity resulting, therefore, in the ablation or significant reduction of proliferating cytotoxic T-cells. Prolongation of islet allograph survival is induced either by a clonal deletion of T-cells, or by the activation of a specific suppressor T-cell population (29,30). In this respect, it is of interest to note that others have induced a similar immune defect (i.e., suppression of rejection) for the protection of grafts such as the pancreatic islet (31) and the heart (32) by treating recipient animals with a combination of an IL-2 antireceptor monoclonal antibody and CsA.

The importance of the present study relates to the likelihood that this approach to islet transplantation may also be applicable to the grafting of humans, and in particular, of both sexes. Aside from the effects of the immunosuppressive Sertoli cell factor on the immune response, it is encouraging to find that the effect occurred independently of the donor origin of the SEF. The results appeared to be the same whether the SEF donor was MHC-compatible with the host rat or not (Table 1, Groups 5 v. Group 6). The fact that SEF need not be MHC-compatible with the host greatly enhances the clinical applicability of this transplantation protocol. To this end, our investigation seeks to determine whether pre-incubation of Sertoli cells, prior to transplantation, alters the marked beneficial effects of the freshly collected SEF on islet allograft survival. Clearly, it would be more practical if cultured preparations could be used instead of dealing with primary cell isolation for each transplantation event. We are currently determining the success and efficacy of islet/SEF transplantation into other organ sites in both male and female rats.

In summary, we have shown that it is possible to create an immunologically privileged site in an organ other than the testis by means of the transplantation of SEF along with the islets. Indefinite islet allograft survival in the renal subcapsular space required, in addition, a short course of CsA therapy, although sustained immunosuppression was not essential. The data indicate that the SEF, and most likely Sertoli cells (10), retain the capacity to secrete an immunosuppressive factor(s) in an organ site other than the testis, and that the presence of these secretions were neither androgenic nor inhibitory to ovulation in female rats. This was demonstrated by the ability of grafted female rats to carry pregnancies to full term and to nurse their pups successfully.

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EXHIBIT J

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FORMATION OF INSULIN-SECRETING, SERTOLI-ENRICHED TISSUE CONSTRUCTS BY MICROGRAVITY COCULTURE OF ISOLATED PIG ISLETS AND RAT SERTOLI CELLS

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Summary

Pancreatic islets, isolated from neonatal pigs, and Sertoli cells, isolated from prepubertal rats, were cocultured in simulated microgravity utilizing the NASA-developed highly accelerating, rotating vessel (HARV) biochamber. Following 5 d of incubation, three-dimensional Sertoli–islet cell aggregates (SICA) retained the ability to secrete insulin when exposed to elevated glucose. SICA contained FasL-positive Sertoli cells and insulin-positive β-cells randomly organized within the spherical construct. The addition of 1% Matrigel induced the reorganization of aggregates (SICAs formed in the presence of Matrigel [SICAmgs]) showing the peripherialization and epithelialization of Sertoli cells and the centralization of islets in association with lumen-like spaces. The Sertoli cells, but not Matrigel, aided in preserving the structural integrity of HARV-incubated islets. Neither Matrigel nor Sertoli cells appeared to interfere with the ability of SICA or SICAmg to secrete insulin and express FasL.

Key words: tissue constructs; transplantation; local immunoprotection; trophic support; diabetes.

Introduction

Pancreatic islet transplantation therapy has received a great deal of attention both in animal models of diabetes (Horaguchi and Merrell, 1981; Calafiore et al., 1990; Evans et al., 1990; Kaufman et al., 1990; Kneteman et al., 1990; Thompson and Mandel, 1990; Richburg et al., 1999) and in humans (Gray et al., 1984; Korbutt et al., 1997; Kuhn et al., 1985; Kneteman and Rajotte, 1986; Alderson et al., 1987; Scharp et al., 1987; Scharp, 1988; Warnock et al., 1988, 1989; Ricordi et al., 1989; Shapiro et al., 2000), and offers hope for the long-term alleviation of diabetic symptoms. Most of the islet-transplantation protocols reported, however, have met with little success as viable treatment for this devastating disease (Naji et al., 1981; Weringer and Like, 1985; Prowse et al., 1986). One significant and notable exception is the recent report of successful human islet transplantation achieved in systemically immunosuppressed patients with type 1 diabetes (Shapiro et al., 2000). The most desirable treatment option, however, would provide the diabetic recipient with a long-term endogenous source of insulin derived from nonhuman islets. The major weakness of this approach is the need for continuous systemic immunosuppression and trophic support of the graft.

In the course of finding a suitable organ or tissue site for islet transplantation, it was discovered that the relocated abdominal testis provides an extraordinarily safe environment for extended survival of islet grafts and some relief from diabetic complications (Whitmore and Gittles, 1978; Selawry et al., 1985, 1986, 1987, 1989). It is now clear that the immunosuppressive and supportive properties exhibited by the abdominal testis are generated by the organ's Sertoli cell population (Cameron et al., 1990; Selawry and Cameron, 1993) and have, therefore, been utilized in cotransplantation protocols to facilitate the success of islet engraftment (Selawry and Cameron, 1993). The salient features of terminally differentiated Sertoli cells that make them potentially significant transplantation facilitators are: (1) they do not express histocompatibility complex (MHC) class I or II antigens on their cell surfaces (Pollanen et al., 1988), (2) they secrete and/or express trophic and immunosuppresant factors (Selawry et al., 1991; DeCesaris et al., 1992; Skinner, 1993; Bellgrau et al., 1995), (3) they are mitotically quiescent (Gondos et al., 1993), and (4) since they live for the life of the donor (Bardin et al., 1988), they may survive for the life of the recipient host (thereby providing long-term immunoprotection and trophic support for the transplanted tissue or cells).

Although Sertoli cells appear to support isolated islets (Selawry et al., 1996) and to immunoprotect islet grafts as well as might be expected when using systemic immunoprotection, there are inherent problems limiting the usefulness of this facilitation protocol, including the difficulty in utilizing primary isolates and the inability to insure the obligatory close proximity of the two cell types following transplantation.

In order to overcome these limitations, it would be beneficial to have a transplantable, viable, tissue-like aggregate composed of Sertoli cells and islets in which the different cell types retain their differentiated functions—that is, normal insulin secretion by β-cells of the islets and immunoprotection and trophic support by the Sertoli cells.

In this report, we describe the tissue engineering, the morphology

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and the functional assay of a Sertoli–islet cell aggregate (SICA) created by simulated microgravity coculture utilizing the NASA-developed rotating wall bioreactor.

MATERIALS AND METHODS

Sertoli cells were isolated from the testes of Sprague-Dawley (16–19-d-old) rats, and pancreatic islets were harvested from neonatal farm pigs. Isolated and pretreated Sertoli cells and isolated, and preincubated islets were cocultured in simulated microgravity for 5 d, after which the resulting SICA were washed, exposed to elevated glucose, and then fixed and processed for morphological analysis and immunodetection of CD-95 (FasL) ligand and insulin. Some SICAs were utilized in a lymphocytic proliferation assay (data not presented).

Media. All media in this project were prepared immediately prior to use. Sertoli maintenance medium (SMM) was prepared as follows. Dulbecco modified Eagle medium/Ham's F12 nutrient medium (GIBCO BRL, Grand Island, NY) was supplemented with retinol (100 μl/100 ml medium; Sigma Chemical Co., St. Louis, MO), insulin transferrin selenium (ITS) (100 μl/100 ml medium; Collaborative Biomedical Products), sodium bicarbonate as per instructions, and gentamicin sulfate (Sigma).

Islet maintenance medium (IMM) was prepared with Ham's F10 nutrient medium (GIBCO BRL) supplemented with bovine serum albumin (500 mg/100 ml medium, fraction V, Sigma), L-glutamine (2 m*M*, GIBCO BRL), D-glucose (1 m*M*, Sigma), isobutylmethylxanthane (0.05 m*M*, Sigma), nicotinamide (10 m*M*; Fisher Scientific, Pittsburgh, PA), sodium bicarbonate (100 μl/100 ml medium, GIBCO BRL), and gentamicin sulfate (Sigma).

For the preparation of incubation medium (IncM), the medium utilized in simulated microgravity incubation in the highly accelerating, rotating vessel (HARV) bioreactor was the SMM with or without 1% Matrigel (Collaborative Biomedical Products, Bedford, MA). Glucose challenges were performed in SMM not containing ITS.

Isolation and pretreatment of Sertoli cells. As previously described, decapsulated rat testes were subjected to sequential enzymatic treatment at 37° C using 0.25% trypsin (Sigma) and 0.1% collagenase (Sigma, type V) (Cameron et al., 1987). The resulting Sertoli cell aggregates, harvested from 20 rats, were distributed in a volume of 50.0 ml of SMM into T150 tissue culture flasks (Costar, Cambridge, MA). Plated Sertoli cell aggregates were incubated in SMM at 39° C in 5% CO_2/95% air for 48 h, after which they were subjected to hypotonic treatment with sterile 0.5 m*M* Tris–HCl buffer for 1 min to expedite the removal of the contaminating germ cells (Galdieri et al., 1981). Following two washes with SMM, the tissue culture flasks were replenished with fresh SMM and returned to the incubator at 37° C in 5% CO_2/95% air. The resulting pretreated Sertoli-enriched monocultures contained greater than 95% Sertoli cells. The plating density ($<2.0 \times 10^6$ Sertoli cells/cm²) did not result in a confluent monolayer of cells.

Isolation and pretreatment of pancreatic islets. Islet isolation was modified from a previous report (London et al., 1990). The pancreas was surgically removed from neonatal (2–4-d-old) piglets, washed (3×) in Hank's balanced salt solution (HBSS), and then minced prior to incubation in 0.20% collagenase (Sigma, type IV) for 17 min. The digested tissue was shaken gently for 1 min, washed (3× HBSS), and pushed through a 50-μm metal sieve. The isolated islets with attached pancreatic exocrine cells were washed (3× HBS at unit gravity) and resuspended into 100 ml of IMM. These islets were incubated in IMM at 38° C and 5% CO_2/95% air for 9 d, replacing media every 48 h, to expedite the gentle removal of the contaminating exocrine cells, while maintaining the structural integrity of the islets. The resulting islet culture was washed (3× IMM) to yield pretreated islets suitable for coculture with the pretreated Sertoli cells. There were less than 5% exocrine cells present, and there was greater that 97% islet cell viability.

Viability, β-cell staining, and coculture. Immediately prior to coculture, cellular viability was estimated by the vital dye exclusion of Trypan blue in the pretreated Sertoli cells and the preincubated islets. The presence of β-cells was determined by diphenylthiocarbozone (DTC). Islets from one half of a pancreas (approx. 15×10^3 islets) were incubated alone or combined with Sertoli cells from three rat pups (approx. 60×10^6 cells) in a volume of 10 ml of IncM, and placed in a 10-ml HARV rotating wall biochamber in the incubator at 37° C and 5% CO_2/95% air. HARV Sertoli–islet cocultures or HARV monocultures (i.e., islets alone) were incubated for up to 14 d with a change of medium after 48-h incubation, and as needed thereafter to maintain normal pH. The rotational speed of the biochamber varied as required, to maintain the rotational suspension of the aggregates.

Glucose challenge and insulin radioimmunoassay. The high-glucose IncM (180 mg%) was replaced with low-glucose (90 mg%) IncM in the HARV 24 h prior to the glucose challenge, after which all SICAs [illegible] were removed and distributed into 35-mm tissue culture dishes [illegible] 5 ml of fresh low-glucose IncM. This medium was collected after [illegible] and immediately frozen to serve as the baseline (time 0) sample. [illegible] were replenished with 5 ml of high-glucose (190 mg%) IncM and returned to the 37° C incubator. Media samples (0.5 ml) were collected every 10 min for 60–90 min, immediately frozen, and later assayed for insulin concentration by double-antibody radioimmunoassay (RIA).

Anti-porcine insulin (lot no. 024H4815; Sigma) was diluted to 1:25,000, using 1:400 normal guinea pig serum. Unknown samples of media (100 μl) were diluted to a final volume of 500 μl with 1% chicken egg [illegible] phosphate-buffered saline (PBS) (pH = 7.5), prior to the addition of [illegible] antibody (200 μl). Porcine insulin (Sigma) was used as a standard, and 100 μl of IncM was added to each standard curve tube. After a 24-h incubation at refrigerator temperature, approx. 0.1 ng of radioiodinated porcine insulin (NEN) was added for an additional 24 h, followed by addition of the second antibody (1:80 anti-guinea pig IgG; Antibodies, Inc., Davis, CA). All samples from a given experiment were run in the same assay. IncM and media with 1% Matrigel (MG) were included as media controls.

Morphology. All SICA were washed (3× IncM) following glucose challenge and fixed for 20 min with 3.0% glutaraldehyde in IncM. Following washes with Dulbecco's PBS (dPBS), some SICA were postfixed with 1% osmium tetroxide in dPBS, washed (3× dPBS), dehydrated in a graded series of ethyl alcohol, and either processed into Epon/Araldite for light microscopy (LM) and transmission electron microscopy (TEM) or dried with hexamethyldisilazane and routinely processed for scanning electron microscopy (SEM). Thick sections (0.5 μm) were stained with toluidine blue for LM, and thin sections (gold/silver) were routinely stained with uranyl acetate/lead citrate for TEM.

FasL and insulin immunostaining. Some fixed SICAs were washed (3× ddH_2O), frozen in liquid nitrogen, and processed into TBS tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC). Cryosections (10 μm) were mounted on glass slides and immunostained for either FasL to identify Sertoli cells or insulin to identify β-cells. Prior to immunostaining, sectioned SICA tissue was treated with 50 mm sodium citrate (pH 8.5–9.0) at 80° C for 30 min to enhance the retrieval of fixed antigens (Jiao et al., 1999). Primary antibodies for FasL (1:50, rabbit polyclonal; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) and insulin (1:500, mouse monoclonal, Santa Cruz Technologies) were processed with the appropriate ABC VectaStain kit (Vector Laboratories, Burlingame, CA), followed by either diaminobenzidene or alkaline phosphatase (Vector) colorimetric markers.

RESULTS

HARV cultures of isolated pig islets alone and HARV cocultures of rat Sertoli cells plus pig islets were incubated in medium with or without 1% Matrigel. Islets best retained their integrity when incubated with Sertoli cells in coculture. Otherwise, islets alone, incubated with or without 1% Matrigel, tended to disaggregate into single cells by 14-d incubation, although cellular viability remained high (>90%), and β-cells remained present as indicated by positive DTC staining.

In the first 24–48 h of HARV coculture, large aggregates appeared in all the biochambers. By day 4, the aggregates had reorganized into smaller and more homogeneous spherical structures that did not change in gross morphology up to day 14 of the incubation period. Aggregates that formed in media containing 1% Matrigel were noticeably larger (3–7 mm) than the smaller ones (0.5–3 mm) that formed in media not containing Matrigel (Fig. 1). Since the size and spherical shape of the aggregates appeared to stabilize by day 4, collection, morphology, and assay of the formed aggregates were performed on those harvested following 5 d of incubation.

Morphology. Following 5 d of incubation, the formed aggregates were generally spherical in shape and, when viewed by SEM,

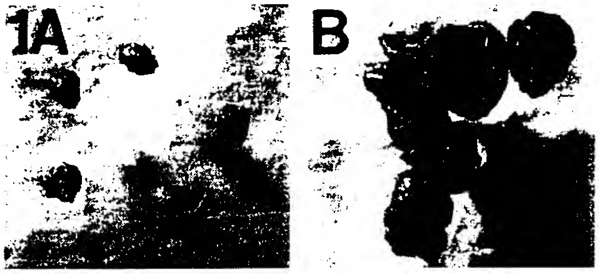

FIG. 1. Rat Sertoli cells coincubated with pig islets in simulated microgravity in defined medium (*A*) result in well-formed aggregates called SICA which are noticeably larger (*B*) when the medium contains 1% Matrigel (SICAmg). Magnification: osmium tetroxide, ×6

showed intact islets surrounded by large Sertoli cells (Fig. 2). Aggregates formed in the presence of 1% Matrigel were not only larger than those from Matrigel-free medium but appeared to be composed of nonislet cells that had a more elaborate structural association with the islets (Fig. 2). The sectional morphology of the fixed aggregates, collected from cocultures with or without Matrigel, identified the components as Sertoli cells and islets, both of which appeared morphologically normal. In the absence of Matrigel, SICA contained randomly organized Sertoli cells that were closely associated with islets (Figs. 3 and 4). SICAs formed in the presence of Matrigel (SICAmg) acquired a more organized morphology when compared with SICA harvested from cocultures not containing Matrigel (Figs. 3 and 4). In the SICAmg, many Sertoli cells were prominently distributed at the periphery of the aggregate and appeared highly polarized (Figs. 3 and 4). Nuclei were positioned basally in the cell, and a distinctive apical cytoplasmic region was oriented toward the center of the SICAmg (Figs. 3 and 4). Sertoli cells at the periphery were in contact with the surrounding Matrigel and were not, typically, in contact with other cells apically. These Sertoli cells were similar in appearance to a simple columnar epithelium, not unlike the structure and organization of the Sertoli cells observed in the intact testis. Additionally, spaces resembling the lumina of seminiferous tubules were present within the SICAmg (Fig. 3). In SICAmg, but not in SICA, the islets, which appeared segregated from Sertoli cells, were more centrally positioned and were in close association with the luminal spaces (Fig. 3). They appeared to have a single layer of attenuated Sertoli cells surrounding them (Fig. 3). The ultrastructure of β-cells and other islet cells in SICA and SICAmg was consistent with the normal ultrastructure of these cells in situ (Fig. 5).

Glucose challenge. All the cells, islets, and/or aggregates (SICA) from a single HARV biochamber were collected and assayed for insulin, following a glucose challenge as described above (Figs. 6–8). It is assumed that all the islets and Sertoli cells originally plated in the HARV, as described above, were viable at the time of assay since there was no evidence of cell attrition based on vital dye-exclusion analysis performed immediately prior to the glucose challenge. Since multiple aliquots from one biochamber could not be considered as separated treatment samples, and since each primary cell/islet isolation necessary to supply the HARV incubation was considered a separate experiment, the results were expressed without statistical analysis.

Islets alone, with or without Matrigel, retained their ability to secrete insulin when challenged with elevated glucose (180 mg%), as indicated by the results of RIA (Fig. 7). When incubated in the presence of Matrigel, the islets alone appeared more responsive to the elevated glucose in terms of insulin secretion when compared with the islets incubated without Matrigel (Fig. 7). Likewise, SICA collected from HARV cocultures, incubated with or without Matrigel, retained the ability to secrete insulin following exposure to elevated glucose (180 mg%) (Fig. 8). Aggregates incubated in media containing 1% Matrigel (SICAmg) appeared to secrete substantially more insulin than did the aggregates incubated without Matrigel

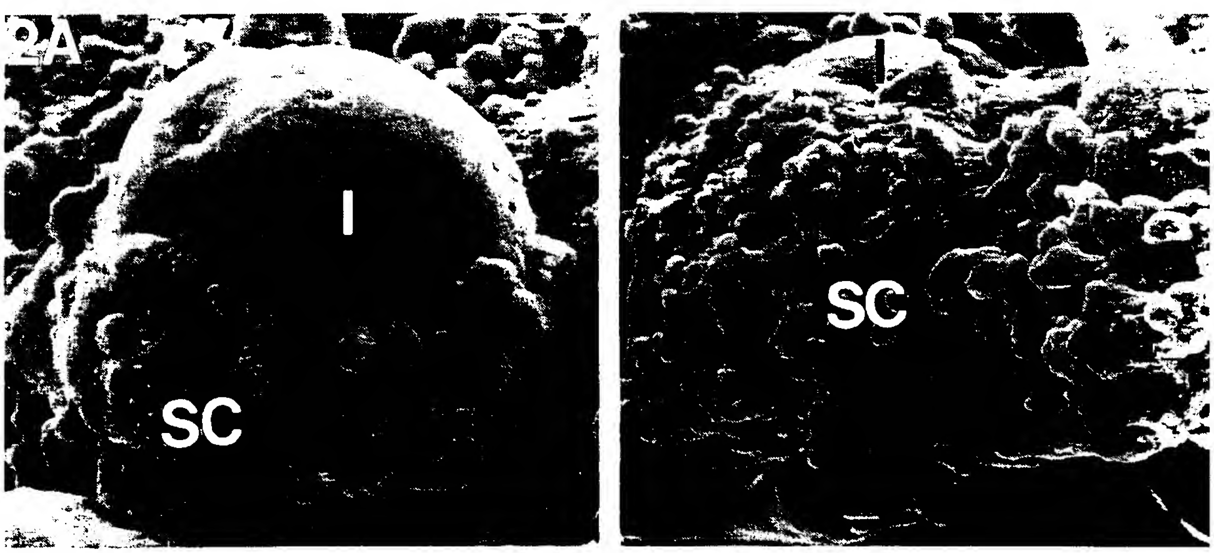

FIG. 2. The high-resolution surface structure (SEM) of SICA (*A*) shows an islet (I) with some Sertoli cells (SC) in close association. The SEM of SICAmg (*B*) shows an islet with a more inclusive association by Sertoli cells of more elaborate morphology. Magnification ×780.

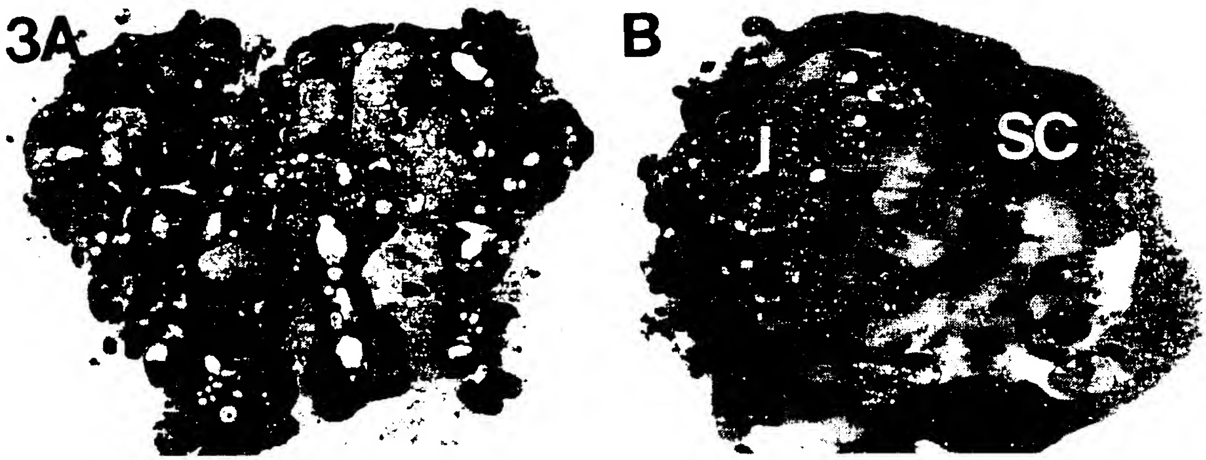

FIG. 3. Sectional morphology (LM) of SICA (*A*) shows a random distribution of Sertoli cells and islet cells, whereas in SICAmg (*B*), Sertoli cells (SC) are distributed more to the periphery of the aggregate, and islet cells (I) occupy a more central position, appearing denser and with many secretion granules. Sertoli cells appear to be more polarized than in SICA, and lumen-like spaces are evident. Magnification: ×200.

(Fig. 8). Based on the standard curve for porcine insulin, incubation media with or without 1% Matrigel did not cross-react significantly in the RIA (Fig. 6).

FasL and insulin immunostaining. All cryosections from both SICA and SICAmg contained cells positively immunostained for insulin, presumably β-cells. In SICA, β-cells appeared to be limited to islet-like clusters of cells (Fig. 9*A*), whereas β-cells in SICAmg sections appeared to be more integrated into the surrounding Sertoli cells (Fig. 9*B*). There was absence of immunostaining in all the negative controls. Quantification of insulin-positive cells was not attempted.

There was positive immunostaining for FasL in all cryosections from both SICA and SICAmg. The pattern of immunostaining for FasL was generally random in SICA (Fig. 10*A*), but distinctively peripheral in SICAmg (Fig. 10*B*). There was absence of immunostaining in all the negative controls.

DISCUSSION

Results from this study show that neonatal pig pancreatic islets and prepubertal rat Sertoli cells form into organized three-dimensional aggregates when cocultured in simulated microgravity utilizing the HARV biochamber. Islet-like structures were identified by cross-sectional analysis of the SICA, and β-cells were identified by positive insulin immunostaining. All islet cell types were present in the formed aggregates as identified by the ultrastructure of secretion granules unique to the different islet cell types. Likewise, Sertoli cells were present in all SICAs examined by positive FasL immunostaining and by their unique cellular ultrastructure.

When incubated with 1% Matrigel, there was segregation of the cell types, with Sertoli cells becoming more peripherally distributed and assuming a more polarized morphology and islet cells occupying a more central position and associated with intra-aggregate spaces. The apparent epithelialization of Sertoli cells following Matrigel addition to the HARV IncM was similar to the well-defined effects of Matrigel on Sertoli cell structure in conventional culture (Hadley et al., 1985; Cameron and Muffly, 1991). It is not clear why intra-aggregate spaces appeared following Matrigel treatment, although it is tempting to speculate that the epithelialization of periferalized Sertoli cells included the formation of occlusive cell–cell junctions, thereby inhibiting the outflow of apically secreted Sertoli cell products. The resulting appreciation of intra-aggregated hydrostatic pressure would result in a "lumen" not unlike the process by which semineferous tubule lumina are formed in vivo (Hadley et al., 1985).

Whether formed in the presence of Matrigel (SICAmg) or without Matrigel (SICA), the aggregates secreted insulin following exposure to elevated glucose (180 mg%). When compared with the controls, insulin secretion was enhanced when 1% Matrigel and Sertoli cells were both present in the coincubation medium. Sertoli cells alone did not appear to enhance the ability of the islets to secrete insulin when compared with controls, whereas Matrigel alone appeared to increase the sensitivity of the islets to the elevated glucose. It is possible that the apparent improvement in insulin secretion from SICAmg compared with SICA was due to the direct stimulatory effects of Matrigel on the islets. It is also possible that the Sertoli cells stimulated islet function by their secretion of positive trophic factors, which was realized only after Sertoli cells themselves had been stimulated by the Matrigel. The direct and/or indirect effects of Matrigel on SICA insulin secretion cannot be determined by the results of this project, and clearly warrants further investigation. Since the design of this project did not allow for multiple sampling from different culture preparations (i.e., all the assayed material came from one HARV bioreactor/treatment group), the statistical evaluation of the functional results was not possible. Therefore, these qualitative observations serve only to indicate an appropriate direction for further study. All the same, it is clear that both SICA and SICAmg tissue constructs retained the ability to secrete insulin and responded to elevated glucose.

It has been known for some time that the pancreatic islets function as a mini-organ. Paracrine interactions among the glucagons-secreting α-cells, the insulin-secreting β-cells, and the somatostatin-secreting δ-cells are believed to be needed for optimal glucose

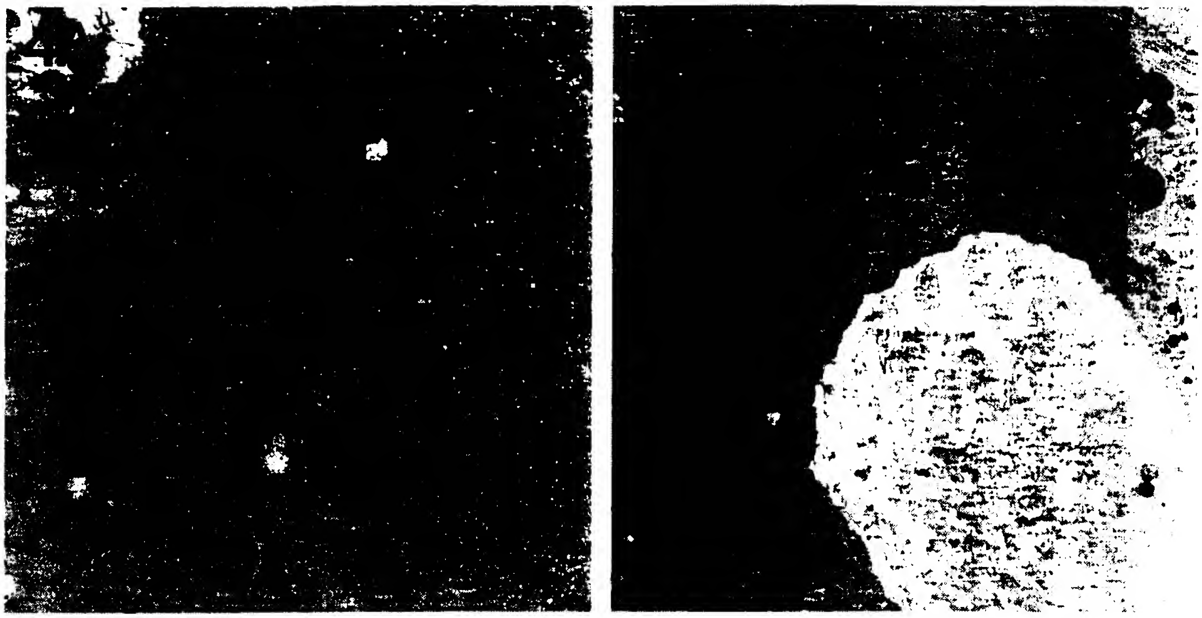

FIG. 4. With high-resolution sectional microscopy (TEM), Sertoli cells in SICA (*A*) and SICAmg (*B*) are dense but organized differently. In SICA, Sertoli nuclei are randomly distributed and without apparent organization. Sertoli cell nuclei in $SICA_{MG}$ are highly organized and appear to occupy a basal position in the cell. A distinct apical cytoplasmic compartment is present which faces a lumen-like space. Magnification: ×6000.

FIG. 5. With TEM, different types of islet cells in SICA can be identified by the unique ultrastructure of their secretion granules. (*A*) α-cell, (*B*) β-cell, and (*C*) δ-cell. Magnification: ×9000.

homeostasis (Unger and Orci, 1976; Unger et al., 1978). Therefore, transplantation of whole pancreatic islets rather than β-cells alone would seem to offer the best chance of restoring normal glucose regulation to diabetic individuals. By the presence of α-, β-, and δ-cells, pig islets incorporated into the SICA retained the general integrity of this multicell endocrine organ, presumably by the structurally supportive network of the surrounding Sertoli cells. The identification of other islet paracrine factors, such as glucagons and somatostatin, was not attempted in the current study and is clearly required to determine the complete functional integrity of the SICA islets. Islets not incorporated into SICA following HARV coincubation deaggregated, presumably due to the absence of the supportive architecture provided for by the Sertoli cells, which Matrigel alone did not provide. However, the β-cells remained viable since insulin secretion was detected in pelleted cells collected from the HARV coincubation medium.

Neonatal pig islets have been well utilized in a number of transplantation therapies, resulting in the successful normalization of hyperglycemia in animal models of diabetes (Yoon et al., 1999). However, most of these protocols involved transplantation of the isolated islets into systemically immunosuppressed or immunodeficient animals such as the nude mouse. It is apparent that when the issues of immune surveillance and graft rejection are eliminated, islets from neonatal pigs serve as a useful source of donor islets for successful transplantation therapies in experimental animal diabetes. Recently, Yoon et al. (1999) showed that following the transplantation of neonatal pancreatic cell clusters in either diabetic or nondiabetic nude mice, there was striking graft development and increased differentiation of nonendocrine cells to the β-cell phenotype. Following the normalization of glucose in the immunodeficient nude mouse, the grafts consisted almost entirely of β-cells, suggesting the capacity of neonatal pig islet cells to selectively dif-

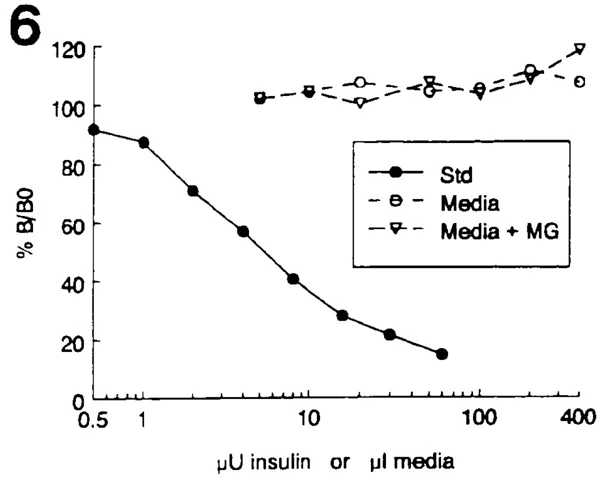

FIG. 6. Typical standard curve for porcine insulin RIA (*solid circles*). Incubation media with (*triangles*) or without (*open circles*). Matrigel (1%) did not cross-react significantly in this assay.

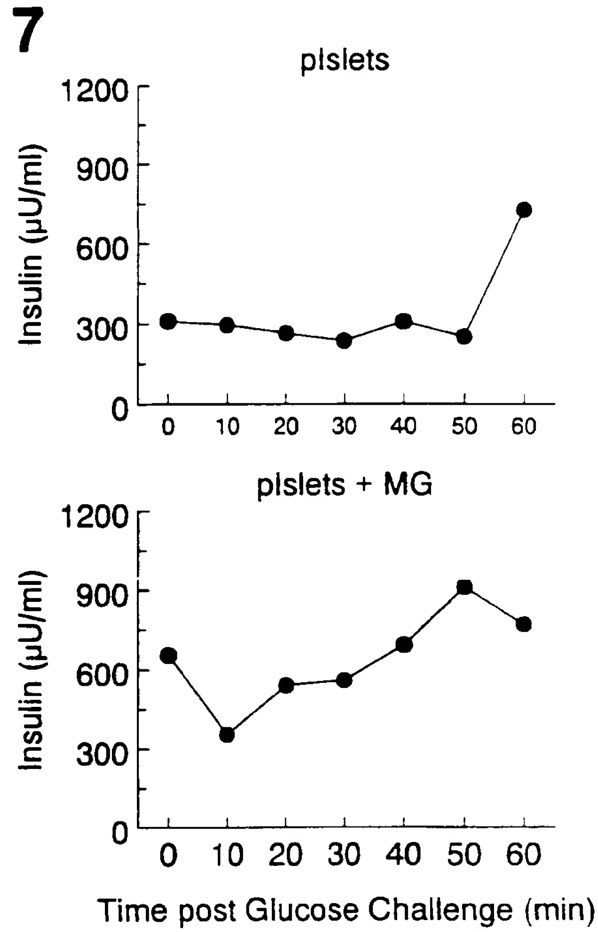

FIG. 7. Insulin secretion (μU/ml media) in response to elevated glucose concentration (180 mg%) of isolated porcine pancreatic islets (15 × 10³) incubated alone or with 1% Matrigel. Each treatment group represented all the cells/aggregates collected from one HARV biochamber.

ferentiate into β-cells (Yoon et al., 1999). Since the maintenance and stimulation of cellular differentiation, not obtainable in conventional culture, has been attributed to the simulated microgravity culture environment (Goodwin et al., 1992, 1993a, 1993b; Schwarz et al., 1992; Becker et al., 1993; Duray et al., 1997), it will be interesting to determine if HARV incubation, with or without Sertoli cells, promotes β-cell differentiation in neonatal islets in vitro.

In the current study, Sertoli cells in all the aggregates examined showed positive FasL immunostaining. It is not surprising that this ligand was detected on SICA and SICAmg Sertoli cells since it has been repeatedly identified on Sertoli cells in vitro and in vivo (Bellgrau et al., 1995; Lee et al., 1999; Richburg et al., 1999; Richburg, 2000). Its expression is significant because FasL has been linked with the immunosuppressive properties of this testicular "nurse" cell (Bellgrau et al., 1995).

Since the report on the utilization of Sertoli cells for the creation of an immunoprivileged site outside the testis, the use of Sertoli cells as a potential immunosuppressive agent has generated considerable interest. Selawry and Cameron (1993) cotransplanted rat Sertoli cells with isolated islets beneath the kidney capsule in the rat. This protocol, when accompanied by a short course (2 d) of cyclosporin A, extended the viability of both the islet allo- and xenograft in the diabetic rat, prevented the rejection of the graft in the otherwise immunologically hostile site, and rendered the once hyperglycemic diabetic rat normoglycemic for the life of the animal.

Although the precise mechanisms by which Sertoli cells or Sertoli cell products affect immunosuppression and trophic support of islets are not yet clear, it is evident that Sertoli cells secrete hundreds of proteins such as insulin-like growth factor–1, transforming growth factor (TGF)α and TGFβ, β–fibroblast growth factor, sulfated glycoproteins 1 and 2, and platelet-derived growth factor (for review, *see* Skinner, 1993). Some are known to have immunosuppressive capabilities, and many are well-defined trophic factors that promote cellular growth and differentiation (Bardin et al., 1988; Skinner, 1993). Several possibilities have been suggested. The expression of FasL may down-regulate the activated T-lymphocyte population by FasL–Fas-induced apoptosis, thereby protecting the graft from the cytotoxic effects of these Fas-positive invading immune cells (Bellgrau et al., 1995). Additionally, Sertoli cells may secrete a protein that suppresses activated T-lymphocytic proliferation by competitive binding of interleukin (IL)-2 receptors on the lymphocytes (Selawry et al., 1991; DeCesarts et al., 1992) and/or suppress lymphocytic IL-2 secretion in conventional culture (Cantrell and Smith, 1984; Selawry et al., 1991; DeCesarts et al., 1992). In this fashion, the putative Sertoli cell–derived immunosuppressant would suppress the rejection by a mechanism similar to the action of cyclosporin A which also suppresses the production of IL-2 (Green and Allison, 1978; Homan et al., 1980; Leapman et al., 1981; Hess, 1985). Coincubation of SICA with ConA-stimulated mixed lymphocytes resulted in the suppression of lymphocytic proliferation (data not shown), similar to the earlier studies reporting the effect of this cell on lymphocytic expansion in conventional culture (Selawry et al., 1991; DeCesarts et al., 1992). These proposed mechanisms, by which Sertoli cells may provide immunosuppression for grafted cells and tissues, are currently under investigation.

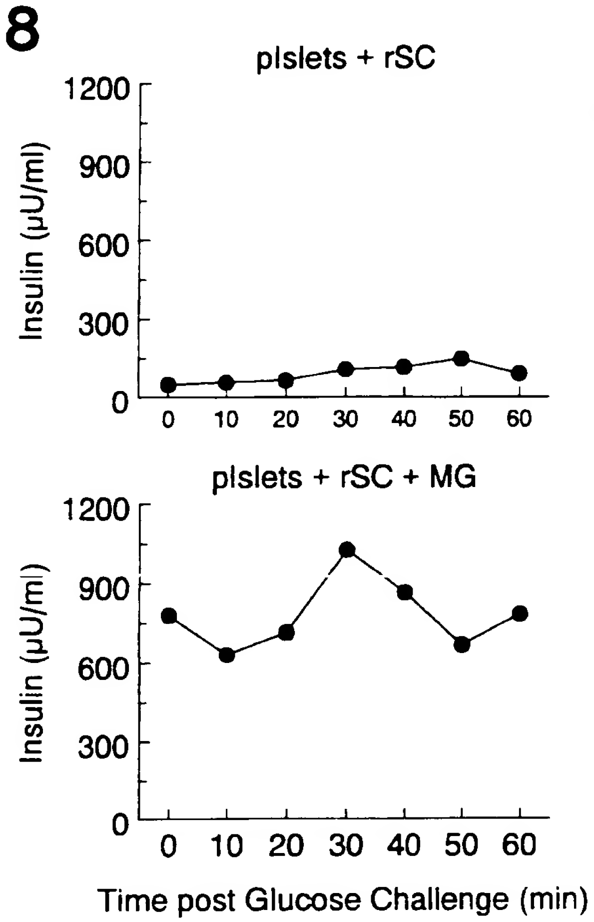

FIG. 8. Insulin secretion (µU/ml media) in response to elevated glucose concentration (180 mg%) of isolated porcine pancreatic islets (15×10^3) following incubation with Sertoli cells (60×10^6) or Sertoli cells plus 1% Matrigel. Each treatment group represented all the cells/aggregates collected from one HARV biochamber.

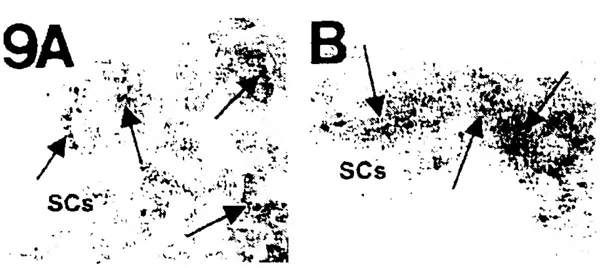

FIG. 9. These cryosections of SICA (*A*) and SICAmg (*B*) are immunostained for insulin to identify the β-cells (*arrows*). *Note* that in the SICAmg (*B*), β-cells (*arrows*) appear to be more integrated into the surrounding Sertoli cell matrix and less confined to the islet structure as observed in the SICA (*A*). Magnification: ×100.

FIG. 10. These sections of SICA (*A*) and SICAmg (*B*) are immunostained for FasL to identify the Sertoli cells (*arrows*). *Note* that in the SICAmg (*B*), the positive reaction product is distinctly distributed to the periphery of the aggregate coincident with the distribution of Sertoli cells. In the SICA (*A*), FasL immunostaining appears more random throughout the aggregate. Magnification: ×100.

The use of Sertoli cells as transplantation facilitators has not been limited to the transplantation of pancreatic islets. The immunosuppresive and positive trophic properties of Sertoli cells have been demonstrated in both allo- and xenografts in the rat brain (Sanberg et al., 1966, 1996; Borlongan et al., 1996; Cameron et al., 1996). It appears that those unique and potentially significant properties of extratesticular Sertoli cells related to successful engraftment of cotransplanted tissue and cells are expressed in both the periphery and the central nervous system.

In conclusion, our results show that the creation of SICA and SICAmg by simulated microgravity coculture of rat Sertoli cells and neonatal pancreatic islets results in a novel tissue construct that exhibits the desirable characteristics of insulin secretion and self-immunoprotection. It is not yet clear to what extent Sertoli cells and Matrigel affect the dynamics of insulin secretion from SICA. The qualitative observations, however, suggest that neither Sertoli cells nor Matrigel interfere significantly with the ability of β-cells to secrete insulin when exposed to elevated glucose in vitro. SICA and SICAmg will be used in experimental long-term transplantation protocols for the treatment of diabetes in experimental models of diabetes.

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EXHIBIT K

Formation of Sertoli Cell–Enriched Tissue Constructs Utilizing Simulated Microgravity Technology

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ABSTRACT: Cell transplantation therapy for diabetes and Parkinson's disease offers hope for long-term alleviation of symptoms. However, successful protocols remain elusive due to obstacles, including rejection and lack of tropic support for the graft. To enhance engraftment, testis-derived postmitotic Sertoli cells have been cotransplanted with islets in the diabetic rat (Db) and neurons in the Parkinsonian rat (PD). Sertoli cell tropic, regulatory, and nutritive factors that nourish and stimulate germ cells also support isolated neurons and islets *in vitro*. Likewise, immunosuppressive properties of Sertoli cells, extant in the testis, are expressed by extratesticular Sertoli cells evidenced by allo- and xenograft immunoprotection of grafts in both the CNS (in the PD model) and the periphery (in the Db model). On this basis, we have created Sertoli islet cell aggregates (SICA) and Sertoli neuron aggregated cells (SNAC) using simulated microgravity culture technology developed by NASA. Isolated rat and pig Sertoli cells were cocultured with neonatal pig islets (SICA) and with immortalized N-Terra-2 (NT2) neurons (SNAC) in the HARV biochamber. Formed aggregates were assayed for desirable functional and structural characteristics. Cell viability in SICA and SNAC exceeded 90% and FasL immunopositive Sertoli cells were present in both. Sertoli cells did not interfere with insulin secretion by SICA and promoted differentiation of NT2 cells to the dopaminergic hNT cell type in SNAC. Addition of Matrigel resulted in structural reorganization of the aggregates and enhanced insulin secretion. We conclude that SICA, SNAC, and Matrigel-induced islet- and neuron-filled "Sertoli cell biochambers" are suitable for long-term transplantation treatment of Db and PD.

KEYWORDS: Sertoli cells; microgravity; HARV; coculture

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INTRODUCTION

Testicular Sertoli Cells

The testicular nurse cell, known as the Sertoli cell, resides in the testis, where it provides a nutrient-rich environment for germ cell expansion and differentiation. Among these proteins are insulin-like growth factor I, basic fibroblast growth factor, transforming growth factors α and β, platelet-derived growth factor, neurturin, interleukin 1α and interleukin 6, and the transport proteins, transferrin and ceruloplasmin.[1] Sertoli cells also protect spermatids from immune detection and destruction, principally by formation of the so-called "blood–testis" barrier, which is, in fact, the collective population of Sertol–Sertoli junctional complexes. Clearly, immunoprotection by testicular Sertoli cells is imparted to the highly antigenic germ cell population. However, it is imparted by mechanisms that are not yet clearly defined, possibly facilitated by the fact that Sertoli cells do not express major histocompatibility complex class I or II antigens and, therefore, may not be detected by the immune system. One suggested mechanism by which Sertoli cells provide immune protection is the constitutive expression of FasL (CD95 ligand). Bellgrau and coworkers[2] showed that the *gld* mouse survived indefinitely when Sertoli cells were transplanted under the kidney capsule. They concluded that the expression of functional FasL by Sertoli cells accounts for the immune-privileged nature of the testis.

Extratesticular Sertoli Cells

Bellgrau and coworkers went further and suggested that FasL expression was the mechanism by which isolated Sertoli cells induce localized immune privilege to cotransplanted cells and tissues,[2] which is appealing in that this is similar to the already well defined mechanism of downregulation of the immune response naturally occurring in the mammalian system. The role of FasL in promoting immunosuppression by extratesticular Sertoli cells in transplantation has received much attention since it was first suggested that Sertoli cells impart their immunoprotective function by this pathway. An alternative or additional mechanism of extratesticular Sertoli cell immunoprotective activity is by way of suppression of activated lymphocytic proliferation. Sertoli cell–conditioned media has been shown to inhibit Con A-stimulated spleen lymphocyte proliferation in a dose-dependent manner. This appears to occur through a dose-dependent inhibition of interleukin 2 (IL-2) production, since the addition of exogenous IL-2 was not able to reverse this effection.[3]

Characteristics of Sertoli cells that make them ideal transplantation facilitators are that (1) they are terminally differentiated and mitotically quiescent when isolated, (2) they live for the life of the donor and may, therefore, live for the life of the host, (3) they do not express MHC I or II antigens, (4) they express and secrete numerous tropic, growth and immunosuppressive factors, and (5) they are easily isolated and cryopreserved

Transplantation Facilitation by Sertoli Cells

In the course of finding a suitable organ or tissue site for islet transplantation, it was discovered that the relocated abdominal testis (at 37°C) provided an extraordinarily safe environment for extended survival of islet grafts and some relief of the

diabetic complications.[4–7] It is now clear that immunosuppressive and supportive properties exhibited by the abdominal testis were generated by the organ's Sertoli cell population[8,9] and, thus, have been used in cotransplantation protocols to "facilitate" the success of islet engraftment.[8,9] The first direct evidence that SCs could, in fact, provide for immunosuppression by a mechanism not involving the B–T barrier was realized when Selawry and Cameron[9] created an abdominal testis-like immunoprivileged site outside of the testis by cotransplanting isolated islets with isolated Sertoli cells beneath to the kidney capsule in the diabetic rat. This protocol, when accompanied with a short two-day course of cyclosporin A, extended the viability of both islet allo- and xenografts, prevented the rejection of the graft in the otherwise immunologically hostile site, and rendered the once hyperglycemic diabetic rat normoglycemic for the life of the animal.[9] Additionally, SCs may facilitate islet graft success by providing for a supportive tropic environment illustrated, in part, by their ability to significantly enhance islet cellular viability following cryopreservation[10] if the islets are thawed in established SC cultures.

On the basis that Sertoli cells might serve as useful "transplantation facilitators" in other cell transplantation protocols, for example Parkinson's disease, isolated SCs were transplanted with rat ventral mesencephalic cells (VM) into the DA-depleted rat striatum, resulting in an increase of graft survival, an increase of surviving TH-positive neurons and an increase of TH-neuron soma size when compared to VM-only grafts.[11] Additionally, Sanberg and coworkers[12] showed that SCs enhanced the survival of bovine adrenal chromaffin cells when cotransplanted in the rat striatum, whereas no chromaffin cells survived in the absence of SCs. Additionally, we have shown that all hNT neuron grafts will survive in the striatum when cotransplanted with SCs without systemic immunosuppression as compared to only 50% survival in hNT-only grafts.[13] This latter observation was accompanied with a 60% reduction of gliosis, indicating a significant suppression of the CNS immune response at the graft site. When transplanted alone, both rat (allografts) and porcine (xenografts) SCs survive in the brain without systemic immunosuppression for ten months post transplantation.[14]

As with islets, SCs provide a supportive tropic environment for neurons illustrated, in part, by their ability to significantly enhance cellular viability following cryopreservation if the VM cells are thawed in established SC cultures.[15] Furthermore, we have shown a significant increase in the numbers of TH-positive neurons in coculture with SCs when compared with neuron-only monocultures.[16] As with the islets studies, Sertoli cells needed to be present to effect these supportive tropic results.

Although extratesticular Sertoli cells support isolated cells and immunoprotect grafts as well as might be expected when using systemic immunoprotection, there are inherent problems limiting the usefulness of this protocol, including the difficulty of insuring the obligatory close proximity of the two cell types following transplantation. To overcome these limitations, it would be beneficial to have a transplantable, viable, tissue-like aggregate composed of Sertoli cells and the transplantable cell type in which the cells types retain their differentiated functions—that is, for example, normal insulin secretion by β-cells of the islets and dopamine production by the neurons and immunoprotection and tropic support by the Sertoli cells.

In this report, we describe the tissue engineering, morphology and functional assay of a Sertoli islet cell aggregate (SICA) and Sertoli neuron aggregated cells (SNAC) created by simulated microgravity coculture using the NASA-developed rotating cell culture system (RCCS).

Simulated Microgravity

The RCCS is a relatively new development in bioreactor technology that enables the cultivation of highly differentiated three-dimensional cell aggregates mimicking the structure and function of parental tissue.[18] The RCCS was originally designed to protect delicate tissue cultures during space flight. However, it quickly became apparent that the unique environment provided by the RCCS of low shear force, high mass transfer, and microgravity, enables three-dimensional cell growth to take place in a conventional tissue culture incubator. The RCCS has a wide range of cell and clinical research applications, including cancer research, *in vitro* toxicology testing, and tissue engineering.

Principle of Rotary Cell Culture

Most culture systems address one specific parameter, shear force, at the expense of others such as mass transfer of nutrients and metabolic wastes, three-dimensionality, and/or cocultivation of dissimilar cell types. The RCCS is the first bioreactor designed to simultaneously integrate cocultivation, low shear, high mass transfer, and three-dimensional growth without sacrificing any other parameter. The RCCS is a zero head space, aqueous medium-filled bioreactor that suspends particles or cell aggregates by rotating the vessel wall and integral gas diffusion membrane around the horizontal axis. The rotation vessel can hold particles/aggregates of up to 1 cm in diameter in orbital suspension, since the sedimentation forces induced by gravity are balanced by the centrifugal force generated by the rotation of the vessel. As the aggregates expand, the rotational speed is increased. Over 40 different cell types have been successfully grown in the RCCS and, to date, no tissue type tested (either suspension or anchorage dependent cells) has failed to grow in the RCCS. For example, hepatocytes have been expanded in RCCS into high fidelity models of liver tissue.[21] Human cancer cells grown include those of melanoma, prostate cancer, breast cancer, ovarian cancer, and osteosarcoma. As an example, normal human fibroblasts have been cocultured with human colon cancer cells and, in less than a month, the coculture producing hundreds of differentiated colon cancer polyps, each exceeding one centimeter in diameter.

To our knowledge, our work is the first attempt to develop Sertoli cell–enriched tissue constructs with highly differentiated cell types suitable for transplantation. This report summarizes our results relating to the creation and definition of SICA and SNAC intended to be used in cell transplantation therapies of experimental diabetes and Parkinson's disease, respectively. The type of RCCS used to create SICA and SNAC was the high aspect ratio vessel (HARV). It is a 10-ml cylindrical RCCS bioreactor with a variable speed power supply. Cocultures were placed in the HARV and then the HARV was placed in a convention incubator at 37°C and 5% CO_2–95% air. The methods for isolating cells and the use of HARV coculture reported herein are described in detail elsewhere.[17]

RESULTS

The detailed morphology and selective assay of SICA and SNAC are reported elsewhere.[17] Presented herein is a summary of those results.

SICA

SICA were formed in the HARV following five days coincubation of isolated neonatal pig Sertoli cells and pancreatic islets. They ranged in size between 1–6 mm in diameter, depending on whether or not 1% Matrigel was added to the coculture medium. Consistently, SICA formed in the presence of Matrigel (i.e., $SICA_{MG}$) were larger than those formed in the absence of Matrigel.

Islets were incorporated within the SICA, as verified by sectional morphology (see FIGURE 1) and illustrated with scanning electron microscopy (see FIGURE 2). All islet endocrine cell types were present, as determined by the unique ultrastructure of secretion granules. Only in the presence of Matrigel did Sertoli cells segregate to the periphery of the aggregate, leaving the islet cells more centrally located and closely associated with lumen-like spaces. In the latter, Sertoli cells were highly polarized and gave the appearance of a simple columnar epithelium, similar to their morphological and histological appearance *in situ*. This phenomenon of Sertoli cell epithelialization *in vitro* has been observed in conventional culture when Sertoli cells are plated on a Martigel substrate,[19,20] but this is the first time that Sertoli cells have been reported to undergo such a dramatic cytoskeletal reorganization when not in direct contact with the substratum.

Cryosections of fixed SICA showed the expression of FasL on Sertoli cells and insulin in β cells by positive immunostaining. In $SICA_{MG}$ there appeared to be an

FIGURE 1. When sectioned (0.5 μm), SICA reveal Sertoli cells (*SC*) surrounding intact islets (*I*). Toluidine blue stain. *Bar*, 100 μm.

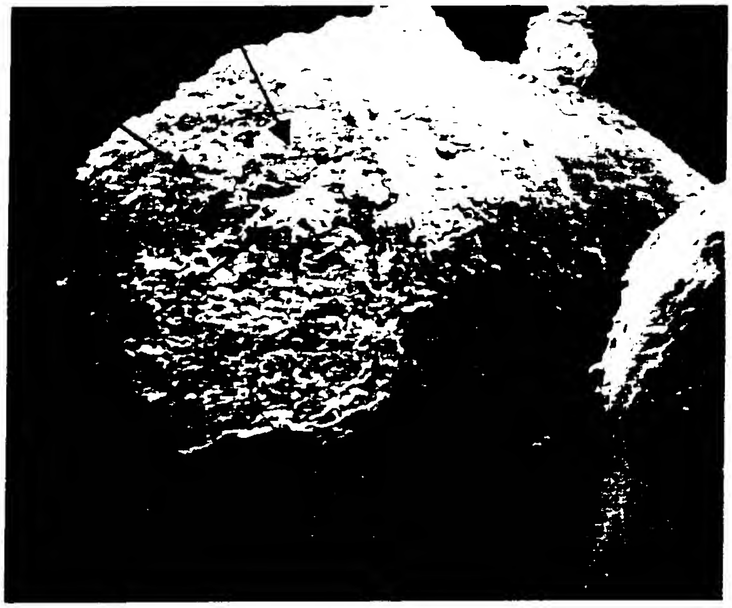

FIGURE 2. This scanning electron micrograph shows a SICA partially fractured. Islets (*arrows*) are present within a matrix of Sertoli cells. *Bar*, 100 μm.

increased density of β cells, although this was not quantified. When exposed to elevated glucose (180 mg%), SICA were capable of secreting insulin as determined by elevated RIA detectable insulin in the medium (see FIGURE 3). Additionally, isolated SICA were capable of suppressing Con-A–stimulated lymphocytic proliferation *in vitro* when compared to islet cell–only coincubation with the mixed lymphocytes.

SNAC

SNAC measuring 1–3 mm in diameter were formed in the HARV following one-week coincubation of isolated rat prepubertal Sertoli cells and the NTerra2 (Nt2) neuron precursor cell line generously supplied by Layton Bioscience, Inc. The NT2 cell acquires a dopaminergic phenotype, illustrated by positive tyrosine hydroxylase (TH) immunostaining, following five weeks of exposure to retinoic acid *in vitro*. As with SICA, Sertoli cell segregation and lumen formation could be induced by adding 1% Matigel to the coculture medium, as demonstrated, in this case, by the peripheral distribution of FasL and centralization of NuMa-positive NT2 cells. The most striking observation of these aggregates was the appearance of TH-positive cells in cryosectioned SNAC, since *in vitro* formation required only one-week incubation, and in the absence of retinoic acid. It would appear that the bioreactor environment of simulated microgravity and/or Sertoli cells accelerated the conditions necessary for dopaminergic differentiation by a mechanism not requiring the retinoic acid.

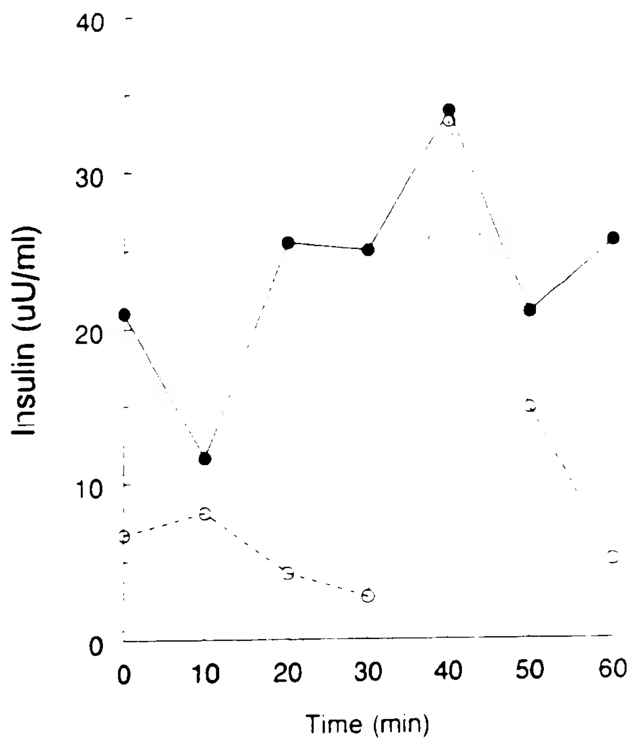

FIGURE 3. This graph illustrates insulin production against time as measured by RIA following exposure to elevated glucose (180 mg%) by isolated SICA (*solid line*), islets alone (*dashed line*), and in medium alone (*dotted line*).

DISCUSSION

Results from this study showed that when cocultured in simulated microgravity utilizing the HARV biochamber, organized three-dimensional aggregates form, following coculture of Sertoli cells with neonatal pig islets (SICA) and NT2 cells (SNAC). In both cases, the transplantable cells exhibited desirable characteristics such as insulin production by β cells in the SICA and TH expression in SNAC neurons. Both prepubertal rat Sertoli cells and neonatal pig Sertoli cells were used in the formation of aggregates, but it is not clear whether one cell type was better than the other in inducing desirable characteristics in SICA or SNAC since the observations were not quantified. Whether or not these novel tissue constructs will enhance the long-term success of cell transplantation therapies for the therapeutic treatment of experimental diabetes and Parkinson's disease also is yet to be determined. However, they do provide a new and promising approach to the ever-growing armamentaria of methods applied to the amelioration of devastating symptoms of these two serious diseases, in particular, and to a number of conditions and diseases involving the replacement of dysfunctional cells, in general.

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EXHIBIT L

BIOLOGY OF REPRODUCTION 14, 481–488 (1976)

Respiratory Gas Tensions in Tissues and Fluids of the Male Rat Reproductive Tract[1]

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ABSTRACT

Micro oxygen electrodes were used to measure PO_2 gradients in rat testes and reproductive tracts. PO_2 (mean mm Hg ± SD) in 14 rats was 15.2 ± 3.5 in interstitial tissue, 12.1 ± 3.3 in tubular lumina, 18.1 ± 5.4 in rete testes, 23.2 ± 2.5 and 24.3 ± 1.3, respectively, in caput and cauda epididymides and 23.1 ± 3.6 in vasa deferentia. Mean testicular vein (TV) PO_2 in six similarly anesthetized rats was 31.3 ± 10.6 mm Hg. In 12 conscious rats with a mean testicular blood flow of 25.5 ± 7.0 ml/100 g/min, blood PO_2 was 86.6 ± 4.7 and 53.4 ± 8.5 mm Hg for femoral artery (FA) and TV blood, respectively, yielding estimates for oxygen uptake of 116–148 µℓ O_2/100 mg of testis tissue/h. In five rats (testicular blood flow 19.8 ± 4.8 ml/100 g/min) the blood CO_2 content was 38.6 ± 5.9 and 48.3 ± 2.9 ml/100 ml blood for FA and TV, respectively, yielding a CO_2 production rate of 114 ± 57 µℓ/100 mg testis tissue/h.

INTRODUCTION

Tissue oxygen tension depends on the supply of oxygen from the blood and its removal by metabolic processes. Temperature also affects gas tension and must, in part at least, explain the low testis interstitial tissue and venous blood PO_2 values reported for scrotal mammals (Cross and Silver, 1962; Free and VanDemark, 1968; Massie et al., 1969; Baker and Lindop, 1970). The germinal cells of the testis are generally supposed to be exposed to an oxygen tension even lower than that in the interstitial tissue since they are farthest removed from the capillary bed. This may be particularly true for the testicular spermatozoa in the lumen of the seminiferous tubules, at least until they reach the rete testis where, because of the arrangement of blood vessels (Setchell, 1970), the potential for reoxygenation exists. Further reoxygenation may occur in the epididymis where the peritubular tissue of the caudal segment was consistently more oxygenated (PO_2 = 13–26 mm Hg) than the testis interstitium (PO_2 = 7–15 mm Hg) in ram, rabbit and dog (Cross and Silver, 1962).

The present study was designed to explore the oxygen tension of the male reproductive tract of rat from the interstitial tissue of the testis through the excurrent ducts to the vas deferens. In addition, since most *in vitro* measurements of testis tissue respiration have been made at nonphysiologic temperatures and gas tensions and may not reflect the true metabolic rate of that tissue, an estimate of respiration rate *in vivo* was attempted by measuring femoral artery and testis vein gas tensions and CO_2 content while monitoring blood flow through the testes of conscious rats.

MATERIALS AND METHODS

Polarographic Apparatus and Calibration Techniques

The polarographic electrodes consisted of Transidyne #721 platinum wire (Transidyne General Corp., Ann Arbor, Michigan) in borosilicate glass oxygen microelectrodes (2-µ tip diameter), and #315 chlorided silver wire reference electrodes. Prior to use the microelectrodes were immersed in 48 percent hydrofluoric acid to expose more platinum. This treatment resulted in a more stable output during usage and calibration and permitted more measurements with each animal preparation. Unbroken microelectrodes were reused after cleaning with acid dichromate solution and rinsing in phosphate buffer followed by distilled water (Inch, 1958). Broken microelectrodes were renovated by cleaning, firing to fuse the tips and sharpening with diamond grinding compound to 20-µ or less diameter tips. These larger microelectrodes were generally used for measurements in the interstitium, caput and cauda epididymis and vas deferens.

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The electrode holders (Fig. 1), fabricated from a disposable pipette and available pin and coaxial connectors, was connected by low-noise coaxial cable through an adapter which provided the −0.6 V polarizing potential, and thence to a Model 610C Electrometer (Keithley Instruments, Inc., Cleveland, Ohio). A Model MP-1001 Console (McKee-Petersen Instruments, Danville, California) containing amplifiers, a millivolt nulling source and a null meter were used to interface the electrometer to an MP-1027 millivolt recorder.

Calibration was carried out in a Transidyne Model 1250 Calibration Cell using physiologic saline and air, 100 percent nitrogen, or an analyzed mixture of 10.09 ± 0.02 percent oxygen in nitrogen. In general, calibration followed the techniques recommended by Transidyne and by Bicher and Knisely (1970). Prior to the experiment the electrodes were preconditioned for about 2 h in saline equilibrated with air, with a −0.8 V polarizing voltage and constant stirring in the calibration cell. The calibration equation was:

$$\text{Partial pressure } O_2 = \%\, O_2\, (P_{(\text{atmospheric})} - P_{H_2O\,(34.5°C)})$$

To ensure the greatest possible accuracy and repeatability of the measurements, temperature of the *in vivo* preparation was controlled and simultaneous atmospheric pressure measurements were taken. Samples of saline saturated with the calibration gases were analyzed for dissolved oxygen by the Winkler Method (Winkler, 1914) and for oxygen tension with a YSI Model 53 oxygen monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio) utilizing Clark-type electrodes. The Clark-type electrodes were used to calibrate the microelectrodes. Calibration points were checked with the Clark-type electrode several times during the course of each experiment.

Animals and Experimental Techniques for Tissue PO_2 Measurements

The Sprague-Dawley-derived albino rats used in these experiments were active breeding males weighing from 370 to 600 grams, maintained under controlled temperature (70°F) and light (12 h light/12 h darkness) conditions. Innovar-Vet (Pitman-Moore, Inc.) was administered intramuscularly (0.2 ml/kg) according to the technique of Jones and Simmons (1968), except that dilution was 1:1 in sterile physiologic saline, and atropine sulfate (0.04 mg/kg) was added.

All tissue measurements were carried out between 1800 and 2100 h. One testis of each anesthetized, dorsally recumbent, air-breathing rat was exposed by a 3-cm mediolateral scrotal incision. After placing the rat and supporting platform into the screen (electrical

FIG. 1A. Apparatus and arrangement for tissue pO_2 measurements in the reproductive tract of the male rat. The electrode holder and testis receptacle.

grounding) cage the testis was eased out of the incision, placed in a temperature-controlled (34.5°C) glass receptacle (Fig. 1a) and covered with saline. The reference electrode was placed in the saline solution within the receptacle. A seminiferous tubule was exposed through a 0.5-cm incision in the testicular capsule and a micro manipulator and dissecting microscope were used to insert the electrode into the tubule (Fig. 1a and 1b). From one to four measurements were taken in each tubule.

For measurements in the rete testes, the ductuli efferentes were ligated by the technique of Tuck et al. (1970). This technique improved visualization of the region due to swelling and permitted new (2-μ tip diameter) electrodes to be used for rete fluid PO_2 measurements after first puncturing the testicular capsule with a 8-μ tip diameter tungsten electrode.

All measurements were recorded as current (range: 10^{-7} ampere for largest electrodes to 10^{-18} ampere for the smallest electrodes), and converted to PO_2 by a calibration curve prepared for each experiment.

Blood Gas Measurements

For blood gas measurements in conscious rats, testicular artery friction flow devices and femoral artery catheters were fitted under halothane/oxygen anesthesia as described by Free and Jaffe (1972). The rats were allowed to recover consciousness in a restraining cage. Testicular blood flow, blood pressures, and heart and respiration rates were monitored over 1 to 4-h intervals. When physiologic parameters were steady, one or two samples (100 μℓ) of blood were taken simultaneously from the femoral artery and testicular vein branch. In blood samples from 12 conscious and 6 Innovar-Vet-anesthetized rats, oxygen and carbon dioxide tensions and pH were measured using a Model IL 113-01 Ultra-micro blood-gas analyzer (Instrumentation Laboratories, Inc., Lexington, Massachusetts). Blood samples from five rats were analyzed for CO_2 content using a Natelson microgasometer. Insufficient blood was obtained from the testis vein of four rats due to venous collapse of the small branch vein or catheter blockage.

RESULTS

A typical micro oxygen electrode recording for the seminiferous tubule of anesthetized rat is given in Fig. 2. Oxygen tension analogs remained relatively steady until the electrode was withdrawn, as in the example shown, or until protein "poisoning" of the tip caused a downward drift. Mean recorded oxygen tensions for the testis tissue and various segments

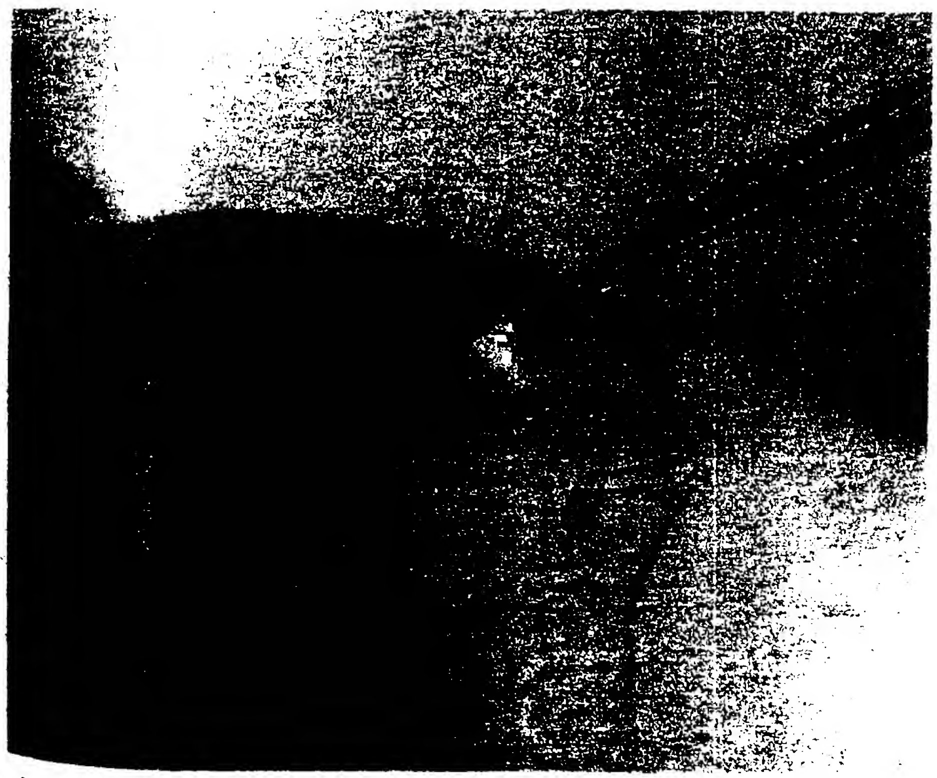

FIG. 1B. Apparatus and arrangement for tissue pO_2 measurements in the reproductive tract of the male rat. The electrode tip in a seminiferous tubule.

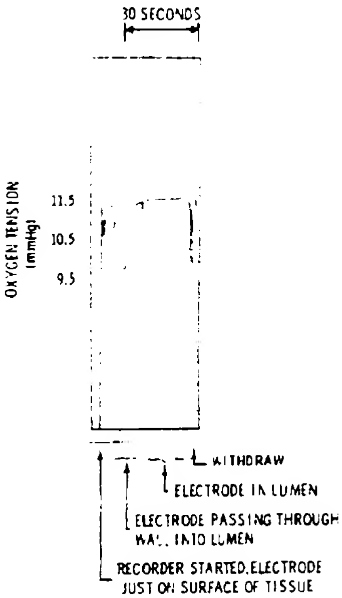

FIG. 2. Microoxygen electrode response to penetration of a rat seminiferous tubule.

of the sperm excurrent pathway are given in Table 1, and show a progressive increase in oxygenation from the lumen of the seminiferous tubules to the lumen of the cauda epididymis. Blood gas tensions and pH for six additional rats, similarly anesthetized, are given in Table 2 and yield mean arterial-venous differences across the testis of 32.4 mm Hg for PO_2 and -8.5 mm Hg for PCO_2.

In four rats, oxygen tension in the interstitial tissue appeared to fluctuate in a rhythmic pattern with a frequency of six to eight per minute (Fig. 3). When the testicular capsule of two rats with rhythmically fluctuating interstitial PO_2 was cut open, the fluctuations ceased and interstitial PO_2 fell rapidly to unmeasurable levels. Hydrostatic pressure (0–100 mm Hg) had no effect on the oxygen microelectrodes *in vitro*.

Testicular blood flow, pressures and heart and respiration rates for 21 conscious rats are given in Table 3 and indicate that the rats were in good physiologic condition when the brief (10-min) sampling period began. Testicular blood flow was monitored during the sampling period and was unaffected by the sampling procedure.

Table 4 shows the mean physiologic gas tensions and pH at 37.5°C for femoral artery

TABLE 1. Oxygen tensions in the testes and reproductive tract of Innovar-Vet[a] anesthetized rats (in mm Hg).

Body weights grams	Interstitial	Intratubular	Rete testis	Epididymis		Vas deferens
				Caput	Cauda	
444	22	19		26		
390	11.5	6	10			
565	16.5	13				
490	19	11.6		22.7		
560	13	13				
540	12.8		26		23.6	26.5
600	14.5	12	17			26
500			21.5			
520		11		21	24.4	
520						20
480		12.5			23	
580		11.8			26	
380	11.7		17			20
540	15.7		17.2			
X	15.2[b]	12.1[c]	18.1[d]	23.2[e]	24.3	23.1[e]
S.D.	3.5	3.3	5.4	2.5	1.3	3.6

[a]Pitman-Moore, Inc

[b]Significantly different from epididymis and vas deferens (Duncan's Multiple Range Test, P<0.05)

[c]Significantly different from rete, epididymis and vas deferens (Duncan's Multiple Range Test, P<0.05).

[d]Significantly different from intratubular, and vas deferens and cauda epididymis (Duncan's Multiple Range Test, P<0.05)

[e]Significantly different from intratubular and interstitial (Duncan's Multiple Range Test, P<0.05)

TABLE 2. Blood gas tensions[a] (in mm Hg) and pH in Innovar-Vet[b] anesthetized rats.

Gram body weight		PO_2	PCO_2	pH
360	TA[c]	56	65	7.26
	TV	20	73	7.20
420	TA	69	61	7.27
	TV	34	69	7.21
450	TA	71	47	7.27
	TV	29	60	7.27
520	TA	71	50	7.02
	TV	29	67	6.88
	FV	50	59	6.93
420	TA	58	55	7.27
	TV	27	58	7.25
	FA	63	62	7.26
410	TA	57	56	7.27
	TV	27	48	7.26
	FA	57	47	7.26
	FV	26	60	7.22
X	TA	63.7	55.7	7.23
	TV	31.3	64.2	7.18
	FA	60	54.5	7.26
	FV	38	59.5	7.08
S.D.	TA	7.4	6.7	0.1
	TV	10.6	6.7	0.15
	FA			
	FV			

[a]Measured at 37.5°C.

[b]Pitman-Moore, Inc.

[c]TA = testicular artery, TV = testicular vein, FA = femoral artery, FV = femoral vein.

and testicular vein blood for 12 conscious rats with a mean blood flow of 25.5 ± 7.0 ml/100 g/min and a mean packed red blood cell volume of 50.5 percent. Mean carbon dioxide content and CO_2 production rates for five conscious rats are given in Table 5.

A respiration rate for rat testis tissue can be estimated from the mean blood flow, hematocrit and oxygen tensions of the 12 rats summarized in Table 3 if a mean corpuscle hemoglobin concentration of 32 percent (Albritton, 1952) and an oxygen-hemoglobin combination factor of 1.39 is assumed. The resulting value for oxygen capacity equals 22.5 ml/100 ml blood. Using a hemoglobin dissociation curve for rat blood at PCO_2 = 40 mm Hg (Jones et al., 1950) a value of 116 $\mu \ell$ O_2/100 mg of testis tissue/h

TABLE 3. Condition of conscious rats at the start of the blood sampling period. (0.5 – 2.0 h postanesthesia; n = 21).

	Mean	± SD
Body weight (g)	469	45
Testis weight (g)	1.81[a]	0.14
Heart rate	410	50
Respiration rate	77	7
Lateral blood pressure (mm Hg)		
Femoral artery	120	6
Testicular artery	61	6
Testicular vein	12.5	1.8
Testicular blood flow (ml/100 g/min)	25.7	7.0

[a]Weight of sampled testis only, measured at sacrifice.

TABLE 4. Blood gas analysis across the testis of the conscious rat (n = 12).

	Femoral artery	Testis vein	Δ
pH[a]	7.39	7.39	0
± SD	0.11	0.11	
PO_2 (mm Hg)[a]	86.6	53.4	33.2
± SD	4.7	8.5	
PCO_2 (mm Hg)[a]	36.2	45.9	9.7
± SD	3.7	4.6	

Mean testicular blood flow for these rats was 25.5 ± 7.0 ml/100 g/min. Mean femoral artery blood hematocrit was 50.5%.

[a]Measured at 37.5°C.

is obtained. Actual CO_2 content measurements in five additional rats (Table 5) yielded a CO_2 production rate of 114 $\mu\ell$ CO_2/100 mg testis tissue/h. Another published dissociation curve for rat hemoglobin (Gjonnes and Schmidt-Nielsen, 1952) yielded an oxygen uptake rate of 148 $\mu\ell$ O_2/100 mg testis tissue/h.

DISCUSSION

Arterial PO_2 values for conscious rats obtained in this study were almost identical to those obtained by Montgomery and Rubin (1974) (83–87 mm Hg) under similar conditions. The PO_2 values for testicular vein blood also compare well with published values (50.4 mm Hg) for barbiturate-anesthetized rats (Schanbacher et al., 1974). Innovar-Vet-anesthetized rats were less well ventilated although arterial-venous differences in gas tension across the testes were similar to those in conscious animals. Since the hemoglobin dissociation curve for rat appears linear over the range 15–85 mm Hg (Jones et al., 1950; Gjonnes and Schmidt-Nielsen, 1952), these

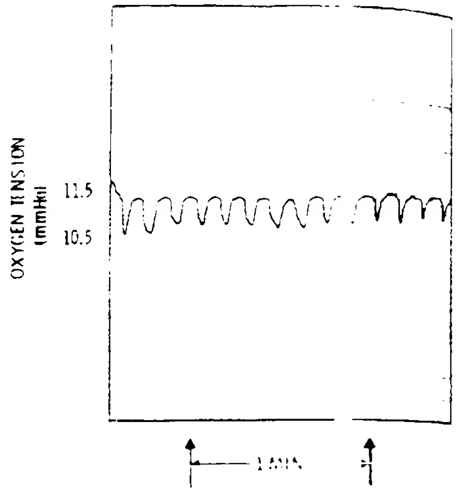

FIG. 3. Rhythmic fluctuation of oxygen tension in the interstitial tissue of a rat testicle.

similar arterial-venous PO_2 changes probably reflect similar oxygen uptake rates.

The mean interstitial tissue PO_2 of 15.2 mm Hg in anesthetized, air-breathing rats was higher than reported values (11.4 mm Hg, Carter et al., 1961; 10.6 mm Hg, Massie et al., 1969). The mean PO_2 value for testicular vein blood of rats under similar conditions is approximately 24 mm Hg when adjusted to *in vivo* testicular temperatures using the correction factors of Nunn et al., 1965. Since the testis has a constant requirement for oxygen and a steady blood flow, the difference in PO_2 between the interstitial tissue and its venous effluent may suggest that some of the testicular blood flow may not pass through the capillary beds of the testis parenchyma, but may pass instead through nonmetabolic channels and remix with the metabolic fraction in the major veins of the testis.

The lumen of the seminiferous tubules is

TABLE 5. Natelson microgasometer measurements of blood CO_2 content across the testis of the conscious rat (9 sample sets from 5 rats).

	CO_2 content (ml/100 ml blood)				
	Femoral artery	Testis vein	Δ	Blood flow ml/100 g/min	CO_2 produced µl/100 mg/hr
Mean	38.6	48.3	9.7	19.8	114
SD	5.9	2.9	4.9	4.8	57

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remote from the blood supply yet does not appear to have a substantially lower oxygen tension than the capillary-rich interstitial tissues. This finding could be explained on the basis of the mixing of the more oxygenated rete and the epididymal fluids with the tubular fluids. Mixing of rete-testis fluid with the fluid formed in the tubules has been suggested to explain the difference in ionic composition between free-flowing and oil-trapped fluid obtained from the rat seminiferous tubules by micropuncture (Tuck et al., 1970). The tubules selected for tissue PO_2 measurement in the present study were always on the surface of the testes and therefore at the periphery of the blood supply. Deeper tubules may have different oxygen tensions, but they are unlikely to be less oxygenated in view of their superior position with respect to the arrangement of blood vessels in the testis (Kormano, 1967).

Rat spermatozoa apparently pass into a progressively more oxygenated environment as they move from the tubule of the testes through the rete, caput and cauda epididymis. A positive oxygen gradient between the testis and epididymis has also been noted in rams, rabbits and dogs (Cross and Silver, 1962).

The 6 to 8 min fluctuations per minute in interstitial tissue oxygen tensions are reminiscent of fluctuation in testicular artery pressure and testicular blood flow seen in earlier studies (Free and Jaffe, 1973). These pressure fluctuations had a frequency of 6/min and also disappeared when the testicular capsule was cut open. *In vitro* and *in vivo* studies with rabbit and human testicular capsules (Davis et al., 1970) have demonstrated an intrinsic contractility with a similar frequency. The effect of these capsular contractions on testicular fluid dynamics is unclear. However, it is possible that they aid in the transport of tubular lumen contents since, being strong enough to affect the vascular bed, they are likely able to exert an effect on the seminiferous tubules. The observations that blood flow remains unaffected when the capsule is cut open (Free and Jaffe, 1972), yet interstitial PO_2 falls to low levels, is puzzling and may suggest that testicular tissue pressure plays a role in the distribution of blood between metabolic and nonmetabolic vessels. Further studies are needed to shed some light on these findings.

Most hemodynamic parameters (Table 3) are similar to those reported earlier for the conscious rat (Free and Jaffe, 1972) although mean blood flow was somewhat higher in the present study.

Previous studies (Free, 1970) have also shown that the *in vitro* rate of oxygen uptake by biopsied samples of rat testis tissue falls off rapidly after separation of the tissue from its blood supply, from an initial value of 70–80 $\mu\ell$ O_2/100 mg/h to 60 $\mu\ell$ O_2/100 mg/h after three hours. These results obtained with oxygen electrodes in air-equilibrated buffer solutions at 33.5°C were only partly dependent on glucose availability, but were entirely dependent on the presence of spermatids and spermatocytes. Most *in vitro* studies of testis tissue respiration have utilized manometric techniques and yielded oxygen uptake rates that are averaged over a 30–60 minute period. This measurement period is usually preceded by varying intervals of preparation and equilibration time, so that actual values obtained for rate of oxygen uptake reflect the metabolic activity of the tissue an hour or more after separation from its blood supply. On the other hand, these manometric studies are usually carried out at body rather than scrotal temperatures so that the decline in respiration due to removal from the blood supply is compensated for, to some extent, by the high incubation temperature. The resulting *in vitro* respiration rates for rat testis tissue have varied from 90 to 98 $\mu\ell$/100 mg testis tissue/h (see Free, 1970, for review). The present *in vivo* estimates, coupled with *in vitro* measurements on biopsied tissue reported previously (Free, 1970) suggest that the rate of oxygen uptake by rat testis tissue is higher than previously supposed.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Eric D. Ordahl for blood gas analysis.

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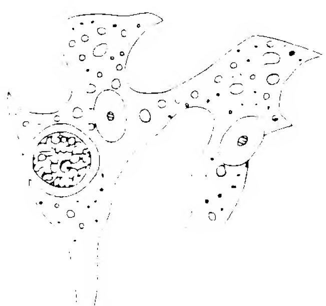
EXHIBIT M

6 Multichamber Cell Culture and Directional Secretion



Drawing (Fig. IVa) modified from Sertoli's original article.

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Polarized Structure and Function of Sertoli Cells *in Vivo*

The structure of the Sertoli cell has been discussed in detail in Chapter 1 and consequently will only be mentioned briefly in relation to those characteristics which are pertinent to polarized secretion. The Sertoli cell is morphologically columnar with the extensively arborized cytoplasm extending from the basement membrane to the lumen of the seminiferous tubule. By virtue of the tight junctions between adjacent Sertoli cells the seminiferous epithelium is compartmentalized into a basal compartment, containing spermatogonia and preleptotene spermatocytes, and an adluminal compartment containing the remaining meiotic and post-meiotic germ cells. It is well established that these Sertoli-Sertoli cell tight junctional complexes form a major component of the blood-testis barrier whereby blood-borne compounds, particularly antibodies, are prevented access to the germ cells within the adluminal compartment. In addition, a less appreciated function of these tight junctions is to prevent Sertoli cell secretory products, from passing between the adluminal compartment and basal compartment. Hence, the tight junctions maintain a polarity of Sertoli cell secretory products some of which are directed apically toward the germ cells in the adluminal compartment and/or the lumen of the seminiferous tubule. Other Sertoli cell secretory products are directed basally toward the germ cells in the basal compartment and/or the interstitium. Due to the complexity of the experimental procedures, studies of bidirectional secretion of Sertoli cell proteins *in vivo* are relatively few in number. Nevertheless, a few studies have established a polarity of Sertoli cell protein secretion from the seminiferous epithelium *in vivo*.

By measuring the concentration of androgen binding protein (ABP) in the testis, epididymis and serum, Gunsalus *et al.* [1] determined that ABP is secreted bidirectionally from Sertoli cells with 80% of this protein occurring in the lumen of the seminiferous tubule and the remaining 20% secreted in a basal direction into the interstitium. Subsequently, these results were corroborated by the micropunture studies of Turner *et al.* [2] which showed that approximately 70% of secreted ABP leaves the testis via the intratubular route. Furthermore, the polarity of ABP secretion from Sertoli cells *in vivo* appears to be influenced by the germ cell therein. For instance, the loss of germ cells in the *grc* mutant rat which exhibits arrest of spermatogenesis after formation of primary spermatocytes [3], partial loss of germ cells in the H^{re} mutant rat [4], and destruction of germ cells by X-irradiation [4] or busulphan treatment [5]; all reduce the apical secretion of ABP in the seminiferous tubule.

Following the initial work of Skinner and Griswold [6] demonstrating that Sertoli cells produce transferrin, Sylvester and Griswold [7] found that seminiferous tubule fluid, rete testis fluid, and testicular lymph contained 141 µg/ml, 47 µg/ml, and 3.7 mg/ml of transferrin, respectively. Using an antibody specifically against Sertoli cell transferrin which does not cross-react with serum transferrin, Morales *et al.* [8] subsequently found that most Sertoli cell transferrin is secreted apically from the seminiferous tubule. By incorporating ^{59}Fe into transferrin or labelling transferrin with ^{125}I, Morales et al. [8] confirmed a model for iron transport into the seminiferous tubule previously proposed by the same laboratory [9]. In this model of iron transport, serum diferric transferrin (holotransferrin) binds to the basal cytoplasm of the Sertoli cells and is endocytosed in coated pits consistent with a receptor-mediated pathway. Subsequently, within the acidic milieu of the intracellular endosomal compartment the iron dissociates from the serum transferrin and becomes incorporated into Sertoli cell transferrin. The serum apotransferrin (lacking iron) recycles out of the basal compartment of the seminiferous epithelium into the interstitium while the iron containing Sertoli cell holotransferrin is secreted apically into the lumen of the tubule and/or bound by germ cells in the adluminal compartment. Hence, in this manner, two forms of transferrin mediate vectorial transport of iron across the Sertoli cell from the basal compartment to the adluminal compartment where the iron is either bound by germ cells and/or secreted into the tubular lumen. (For more detailed discussion of transferrin see Chs. 7, 8 and 13).

Inhibin is known to be secreted by Sertoli cells bidirectionally into the seminiferous tubule lumen and into testicular interstitial fluid [10]. By measuring levels of inhibin in venous blood and interstitial fluid, Maddocks and Sharpe [11] concluded that inhibin is secreted predominantly into the lumen of the seminiferous tubule and is reabsorbed from the rete testis into mediastinal venous blood. Subsequently, these same authors [12] determined that in immature (28-35 day-old) rats approximately 60% of the inhibin-alpha was secreted from the base of the Sertoli cells into the interstitium, whereas in adult rats (45-100 days-old) approximately 95% of the inhibin-alpha was secreted into the lumen of the seminiferous tubule and reabsorbed from the rete testis as previously described [11]. It appears that the route of inhibin secretion in the adult rat is also modulated by germ cells. In this context, following methoxy acetic acid depletion of round spermatids from the rat testis, the apical secretion of immunoactive inhibin is reduced [13], suggesting that germ cells stimulate the adluminal secretion of inhibin into the lumen of the tubules, whereupon it is reabsorbed from the rete testis into the venous blood.

Comparison between Conventional Culture Systems and the Multichamber Culture System

The rationale for the development of the multichamber culture of Sertoli cells was conceived out of the realization that Sertoli cells cultured on plastic do not resemble their *in vivo* counterparts. Indeed, features of Sertoli cells *in vivo* that are lacking in conventional cultures *in vitro*

pertain to the Sertoli cells' polarized structures and functions and their compartmentalization thereof. In order to better mimic Sertoli cell growth *in vitro*, an idealized cell culture system should exhibit polarized cytoplasmic structures that compartmentalize the epithelium. In addition, the cell culture system should exhibit polarized biological functions such as bidirectional endocytosis, polarized secretion, transcellular transport, and the capacity to examine cell-to-cell interactions, all of which are generally not possible or impaired in conventional cultures on plastic.

Differences in the types of endocytosis that occur at either the apical or basal surface of Sertoli cells appear to be a function of the distinctly different environments that surround the Sertoli cell *in vivo*. For instance, phagocytosis occurs mostly from the apical surface where residual bodies are internalized and degraded in the cytoplasm [14, 15]. In contrast, large structures amenable to phagocytosis are much less prevalent along the basal domain of Sertoli cells, presumably due to the filtration effect of the basal lamina and basement membrane underlying the cells. Sertoli cells preferentially, but not exclusively, participate in fluid-phase endocytosis from their apical surface [14, 17]. Nevertheless, it seems likely that most of the nutritional requirements of Sertoli cells are obtained through pinocytosis (fluid-phase endocytosis) of blood-borne compounds that have permeated through the extracellular matrix to the basal surface of the Sertoli cells. Specific proteins such as serum transport proteins (e.g., transferrin) and growth factors require a receptor-mediated process for their internalization and exhibit compartmentalized localization of such receptors along the basal domain of the Sertoli cell [16]. Clearly, fluid-phase and receptor-mediated endocytosis of substances along the basal domain of Sertoli cells is the major route of uptake of compounds for the nutritional requirements of Sertoli cells *in vivo*; however, such a route of uptake is eliminated for Sertoli cells grown on plastic where the cells are forced to internalize substances exclusively from the apical surface.

Polarized secretion *in vivo* of Sertoli cell proteins such as ABP, transferrin, and inhibin has already been discussed above. The physiological significance of the bidirectional secretion of proteins such as ABP and inhibin remain unclear. Indeed, such bidirectional secretion of Sertoli cell proteins may simply represent an imperfect secretory pathway whereby a small proportion of protein is missorted into the incorrect plasma membrane domain. Alternatively, since bidirectional secretion *in vivo* of Sertoli cell ABP and inhibin is influenced by germ cells, it appears that cell-to-cell interactions may participate in the regulation of polarized secretion. In this context, germ cell damage, resulting in a modification to the polarized secretion of some Sertoli cell proteins, may represent a feedback mechanism for the repair of testicular function. In any event, the bidirectional secretion of Sertoli cell proteins *in vivo* cannot be observed in Sertoli cells grown on plastic. This then raises the issue of whether the forced unidirectional secretion of Sertoli cell proteins cultured on plastic further modifies Sertoli cell secretion with respect to quantitative changes in the total secretory protein and qualitative changes in the composition of these proteins. Transcellular transport across Sertoli cells in a basal to apical direction has been demonstrated to occur for iron [7-9] *in vivo*, and most probably occurs for some other elements and compounds that are required by germ cells. Conventional cultures of Sertoli cells on plastic, in the absence of access to the basal domain of the cells, cannot be used to investigate transcellular transport of compounds in either direction; culture of Sertoli cells on a suspended semipermeable support provides the required access to the base of the cells for such studies.

Cell-to-cell interactions *in vivo* occur with the apical domain of the Sertoli cell and the germ cells that have completed or have been committed to meiosis in the adluminal compartment of the seminiferous epithelium. Cell-to-cell interactions between Sertoli cells and germ cells in the basal compartment of the seminiferous epithelium (spermatogonia and preleptotene spermatocytes) as well as the underlying peritubular myoid cells and Leydig cells occur with the basal cytoplasm of the Sertoli cells. Clearly, the spatial arrangement of these latter interactions is difficult, although not impossible to establish *in vitro*. In this context, the multichamber culture of Sertoli cells lends itself to culture of germ cells on top of the Sertoli cell monolayer (Fig. 1B); culture of peritubular myoid cells underneath the permeable support (Fig. 1C); and culture of Leydig cells in the basal reservoir of the chamber further underneath the Sertoli cells and peritubular myoid cells (Fig. 1D). In this manner the architectual and spatial orientation of the major cell types of the testis can be reconstructed in a manner that better mimics their *in vivo* relationship, as shown in Figure 1.

The limitations of conventional culture on plastic whereby only one surface of the Sertoli cell is available to interact with its milieu, as discussed above, provide a strong rationale for the development of a multichamber system that provides access to both surfaces of the Sertoli cell, while at the same time maintaining the compartmentalized disparate milieus that surround the apical and basal surfaces of the cell. Such a multichamber culture system more closely mimics the architectural and spatial relationships of the major testicular cell types *in vitro* (Fig. 1), thereby allowing study of fundamental biological features of the Sertoli cell such as bidirectional secretion and endocytosis, transcellular transport, and cell-to-cell interactions, under highly controlled conditions.

Culture and Characterization of Sertoli Cells in Multichamber Systems

There are several elements that contribute to the formation of a multichamber culture system. Firstly, there are the porous filters which act as a support on which the cells

grow and concurrently provide channels for the movement of nutrients to the basal cytoplasm and, conversely, allow movement of secretory products away from the basal cytoplasm of the cell (Fig. 1 & 2). Secondly, these filters can be coated with either extracts or purified components of extracellular matrix in order to mimic the basement membrane which underlies Sertoli cells *in vivo*. Thirdly, the Sertoli cells which are seeded on the matrix coated filters have been prepared from different aged rats by enzymatic procedures which yield different sized fragments of seminiferous epithelium for seeding in the chambers. Fourthly, the culture media within which the Sertoli cells are cultured varies between laboratories. Considering these four major elements of the filter, extracellular matrix, Sertoli cell preparation, and culture media that contribute to the formation of a multichamber culture system, it is not surprising some results have varied between laboratories.

The types of supports predominantly utilized for the multichamber culture of Sertoli cells have included nitro- and acetylo-cellulose filters from Millipore Corporation (Fig. 2) and polycarbonate filters from Nucleopore Corporation. The first two reports to concurrently describe the multichamber culture system for Sertoli cells used cellulose filters of 1.2 μm pore size [18] and 0.45 μm pore size [19] from Millipore corporation. Many research laboratories have continued to utilize the cellulose filters [20-25, 31, 32, 36, 50], whereas others have changed to the polycarbonate filters [26-28, 51]. Some debate in the literature has centered around the binding of proteins to the various types of filters. Hadley *et al.* [21] found that the non-specific association of proteins such as transferrin and ABP to the Matrigel coated cellulose filter saturated after 8 hours and does not increase any further as a function of protein concentration. Moreover, presaturation of Matrigel coated cellulose filters with Sertoli cell conditioned media was found to prevent substantial binding of ^{35}S-methionine labeled Sertoli cell secretory protein subsequently added to the same support [29]. Since several days of culture elapse between the initial seeding of the Sertoli cells in the chambers and their subsequent use, presaturation of the Matrigel coated filters occurs with Sertoli cell secretory protein during the period of culture prior to experimentation. Janecki and Steinberger

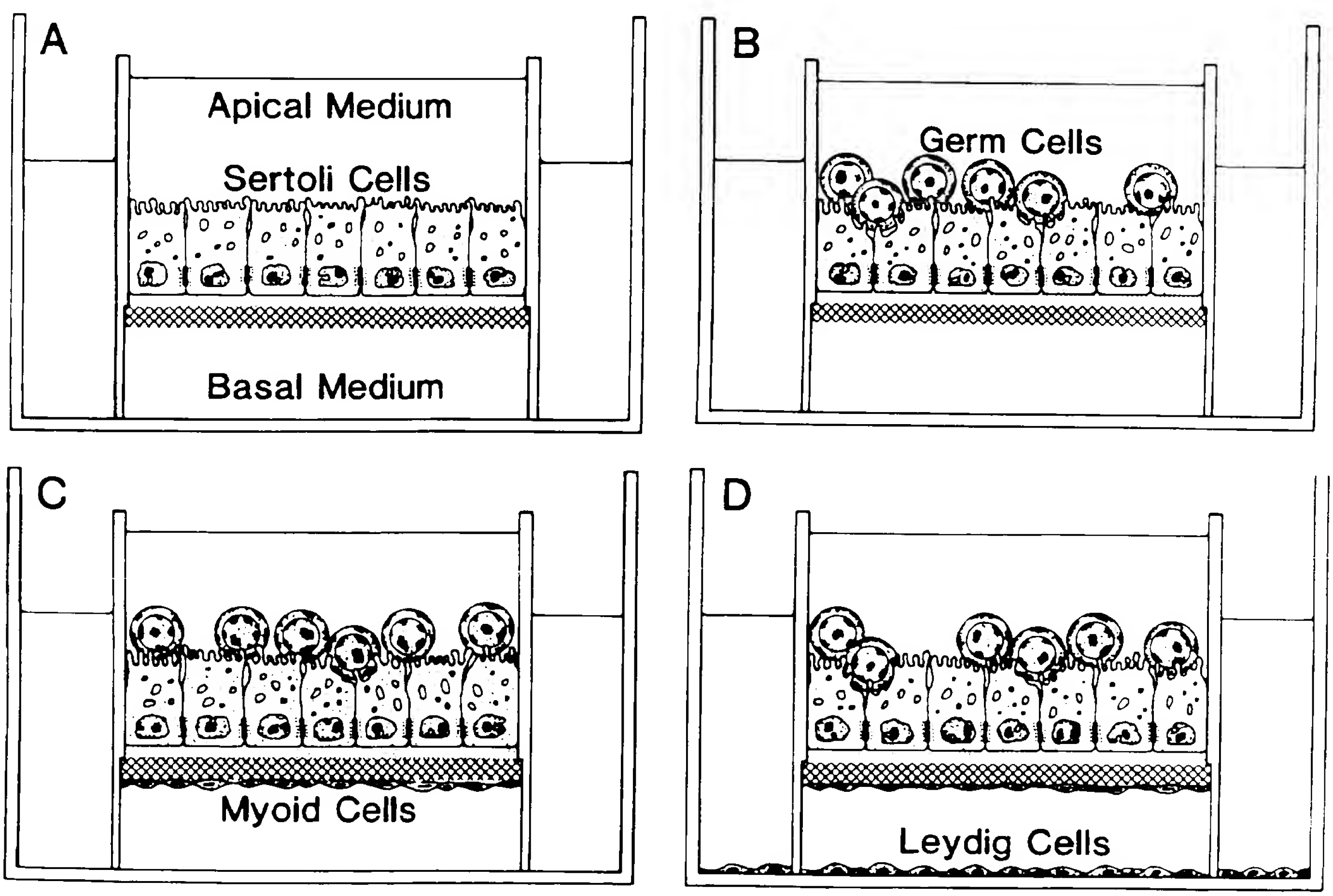


Figure 1. Schematic representations of confluent epithelial sheets of Sertoli cells in multichamber culture systems (A) cocultured with germ cells (B), peritubular myoid cells (C), and Leydig cells (D) in a spatial orientation consistent with the relationship of these cell types *in vivo*.

Figure 2. A photomicrograph of multichamber inserts (Millicell-HA: Millipore Corp.) of two sizes next to the appropriate sized multiwell plates used for culture of Sertoli cells. Small (left) and large (right) multichamber inserts of 12 mm and 30 mm diameter, respectively, are shown.

[26] found that greater than 80% of ABP and transferrin bound to Matrigel-coated cellulose filters was reduced to less than 10% binding when the support had been presaturated with 5% BSA. Binding of ABP and transferrin to polycarbonate filters was reported to be less than 4% of total activity [26]. Curiously, Handelsman *et al.* [24] found that inhibin could pass readily across the cellulose filters, whereas Janecki *et al.* [27] found that it was bound by the same type of filter. Clearly, some binding of protein occurs to the filters; however, the amount of binding is influenced by the degree of pre-saturation of the support and the chemical composition of the filter. Moreover, binding of proteins such as growth factors to extracellular matrix components of the basement membrane has been shown to occur *in vivo*, and is thought to function as a physiological reserve of specific proteins such as fibroblast growth factor [38]. However, within the multichambers the degree of protein binding to extracellular matrix components required to mimic the physiological condition remains to be determined. In any event, for the multichambers there is a reasonable consensus among laboratories that larger proteins such as ABP [21, 26] and transferrin [21, 24, 26] and small compounds such as inulin [24, 31] equilibrate across the cellulose filters coated with or without Matrigel within approximately 12 hours. Studies of equilibration times across polycarbonate filters remain to be reported. Hence, for surety of protein equilibration and convenience of experimentation many studies of polarized secretion using filter supports have been carried out over a 24 hour period, or greater.

Since the work of Hadley *et al.* [30] demonstrating the effect of a reconstituted basement membrane extract (Matrigel) from the EHS mouse tumor cell line on maintenance of Sertoli cell differentiation and secretory function, most laboratories growing Sertoli cells in multichambers have utilized Matrigel as a coat of extracellular matrix for filter supports. Muffly and Hall [32] utilized a fibronectin/collagen IV coat for filter supports. The concentration of Matrigel used to coat the filter supports has varied between laboratories. Matrigel has been applied undiluted [18, 21, 36] at approximately 10 mg/ml and at dilutions of 1:1 [24, 26], 1:2 [20, 22, 36], 1:5 [23], 1:6 [27], and 1:8 [28]. The most recent studies have utilized the more dilute Matrigel, providing some consensus that a dilution of 1:5 to 1:8 is sufficient to coat filters for attachment of Sertoli cells and at the same time providing economical use of reconstituted basement membrane.

Virtually all of the studies of directional secretion in multichambers have been from Sertoli cells derived from the rat, with the exception of one study [25] with baboon (*Papio hamadryas*) Sertoli cells. The age of the rats from which the Sertoli cells are derived for seeding in the chambers has varied from 10 days [18, 20, 21] through 25 days [32]. The Sertoli cells from the 10 day-old rats are relatively difficult to dissociate into small fragments [18, 20] and are less well differentiated, whereas the Sertoli cells from the 25 day-old rats are contaminated with a greater number of germ cells. Hence, most investigators have utilized Sertoli cells from rats of 17-22 days of age [19, 21, 23-29, 31, 33-36] which are readily isolated from the seminiferous epithelium, relatively easy to culture and contain a small proportion of germ cell contaminants. Following several sequential enzymatic digestions to remove peritubular myoid cell contaminants, tubule fragments ranging in size from approximately 100 cells [22] through 10-50 cell aggregates [20, 21, 23, 29], to small aggregates of fewer than 10 cells [23, 28, 37, 51] have been seeded in chambers. A schematic representation of a more recent procedure is shown in Figure 3. The only study to examine the size of the cell aggregates in relation to the characteristics of the Sertoli cell epithelial sheet in the multichambers was carried out by Onoda *et al.* [23]. This study found that small aggregates of up to 10 Sertoli cells seeded in the chambers produced a more even epithelial sheet of Sertoli cells which could maintain a hydrodynamic permeability barrier. The larger cell aggregates did not spread evenly, with multilayers of Sertoli cells on some parts of the filter surface. Poor contact between cell aggregates on other parts of the filter resulted in some leaky chambers.

Plating cell density is also an important consideration in obtaining confluent epithelial sheets of Sertoli cells. Since Sertoli cells derived from approximately 20 day-old rats do not divide to any significant degree, the cells are plated at high density to achieve confluence. In addition, high plating cell density prevents cell spreading by tight packing of the cells, thereby maintaining polarized morphology. Excessively high plating cell densities result in multilayers of Sertoli cells. Hence, the appropriate plating cell density which balances the requirements of high density for confluence and polarized morphology against a plating density which produces an approximate monolayer (more appropriately termed confluent epithelial sheet) needs to be established for each multichamber system. The only study to examine the effects of cell density on optimal formation of a confluent epithelial sheet correlated with formation of a hydrodynamic permeabilty barrier was carried out by Onoda *et al.* [23]. In this study five densities of Sertoli cells ranging from 1.6-5.6 x 10^6 cells/cm^2 were examined (Fig. 4). Onoda *et al.* [23] found

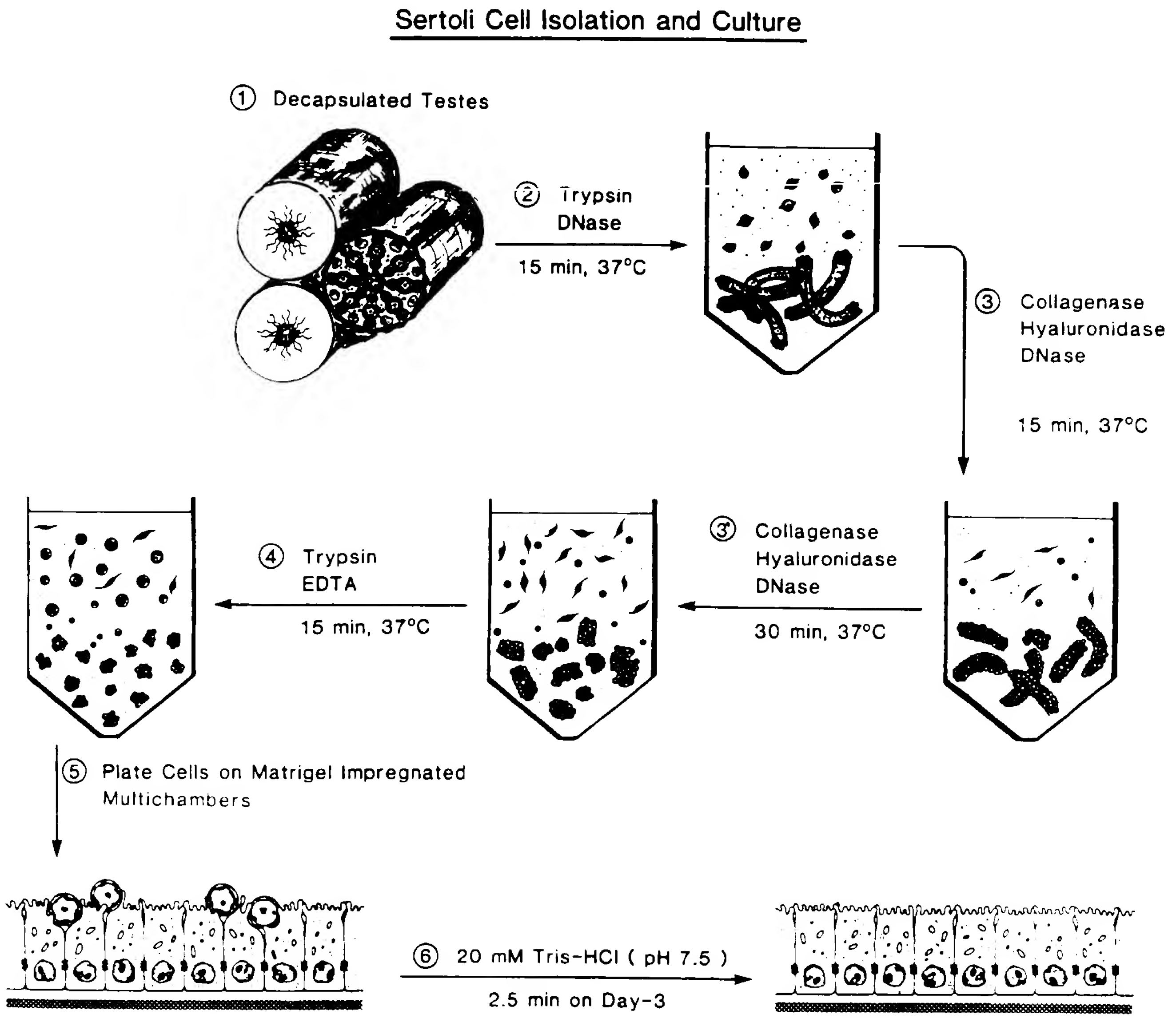


Figure 3. A schematic representation of the isolation and culture of Sertoli cells in multichambers as modified from the procedure of Onoda *et al.* [23] and Onoda and Djakiew [39, 40]. Decapsulated testes (Step 1) are enzymatically digested to remove interstitial tissue (step 2), and further digested to remove peritubular myoid cells (Step 3). This procedure is repeated to fragment the seminiferous tubules (step 3'), which are subsequently treated with an additional enzymatic digestion (Step 4) to produce small aggregates of approximately ten cells or less, which are plated on Matrigel impregnated supports in multichambers (Step 5). After three days in culture the Sertoli cells and remaining germ cells are treated with a hypotonic buffer (Step 6) which lyses the germ cells, leaving a confluent, epithelial sheet of Sertoli cells in monoculture.

that cell densities of 3.8 - 5.6 x 10^6 cells/cm^2 produced polarized confluent epithelial sheets of Sertoli cells which also formed hydrodynamic permeability barriers. Sertoli cell densities within this range have been reported for several other studies [18, 20, 21, 24, 25, 50]. At such high cell densities inulin passage was relatively slow (Fig 5A), electrical resistance was high (Fig. 5B), the polarity of transferrin secretion was in a predominantly apical direction with the ratio (apical/basal) of secretion greater than 1 (Fig. 6), all of which were associated with formation of a hydrodynamic permeability barrier (Fig. 7). Indeed, formation of a hydrodynamic permeability barrier, as indicated by the maintenance of uneven fluid levels in the apical and basal reservoirs of the chambers (Fig. 7), has proven to be a convenient and easy method for assessment of the epithelial sheet of Sertoli cells in the chambers. In this context, at cell densities of 3.1 x 10^6/cm^2 and below, the hydrodynamic permeability barrier formed with variable consistency and the polarity of transferrin secretion was inverted in a predominantly basal direction (Fig. 6). Taking into consideration the characteristics of the Sertoli cell epithelial sheets reported above [23] it is not surprising that the polarity of Sertoli cell secretory proteins has inverted in a predominantly basal direction in those studies which have utilized Sertoli cell densities below 3.1 x 10^6/cm^2 [27, 28, 31, 33-35, 37, 51].

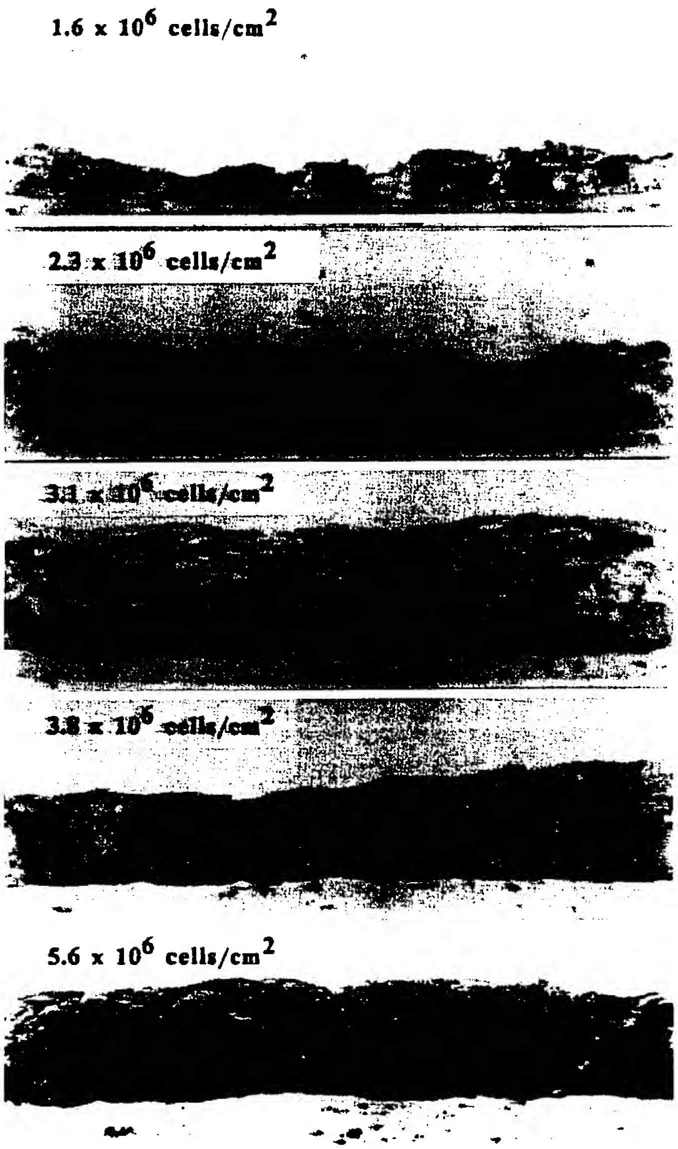


Figure 4. Density dependent polarized morphology of Sertoli cells seeded on Matrigel coated permeable supports (filters) in multichambers. x520. (Modified from Onada *et al.* (1989)[23] reproduced with permission from the Publisher, the Endocrine Society.)

Various serum free defined media formulations [18, 19, 32, 36, 50] as well as serum based media [31, 37] have been used to culture the Sertoli cell epithelial sheets. The rationale for a serum-free defined media is that all constituents are known and generally have been shown to influence Sertoli cell function. In this context, serum varies with each lot and if not properly screened and/or treated may contain cytotoxic components. The most complex serum-free defined media containing 14 supplements was developed by Hadley *et al.* [30] and contains among other components, a number of transport proteins, growth factors, vitamins, hormones, and metabolic substrates. Several defined media formulations from various laboratories [19, 27, 36, 37] contain many of the same components as that of Hadley *et al.* [30] which and are widely used for culturing Sertoli cells [18, 20, 23, 24].

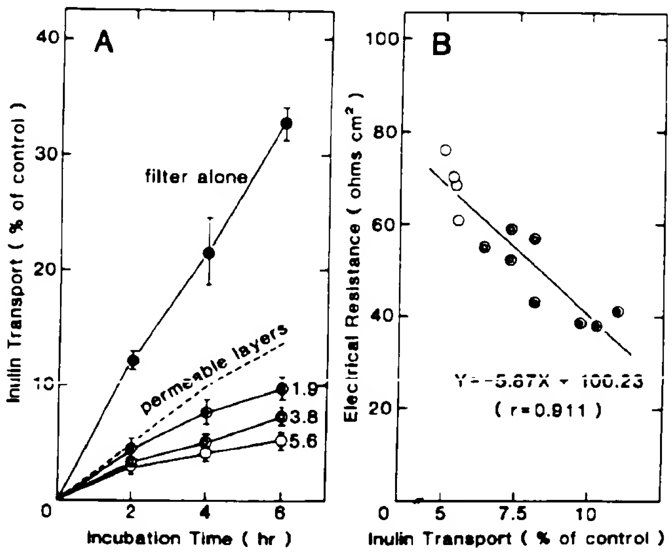
Directional Secretion from Sertoli Cells Cultured in Multichamber Systems

Intuitively, it seems reasonable that Sertoli cell proteins which primarily interact with meiotic and post-meiotic germ cells will be secreted apically, whereas other Sertoli cell proteins may be secreted basally. Some of the compounds that have been shown to be transported-/secreted in a directional manner from Sertoli cells grown in multichambers can be conveniently grouped by their nature and function. The first of the compounds include elements such as Rb^+ [32, 35] and Fe^{2+} [20] which have been shown to be vectorially transported across Sertoli cells within the multichambers. The second group of compounds are Sertoli cell transport proteins such as ABP [21], transferrin [21, 36] and ceruloplasmin [39, 40] which are homologous to serum transport proteins, and are thought to mediate directional transport of testosterone, Fe^{2+}, and Cu^{2+}, respectively. The third group of proteins are proteases [33, 34] which may play a role in remodeling of the seminiferous epithelium. The fourth group of proteins are the endocrine/paracrine factors, such as inhibin [24, 25] and the Leydig cell steroidogenic stimulatory factor [41]. The last group of remaining proteins are not readily categorized and include sulfated glycoprotein-1 (SGP-1)[29, 36, 40], SGP-2/clusterin [29, 36, 39, 40], testins [39], and SP1 [36].

Elements

Rb^+, which is thought to act as an analog of K^+ [32], has been used to mimic K^+ transcellular transport across Sertoli cells. The studies of Muffly and Hall [32] showed that the apical efflux of Rb^+ was not inhibited by azide and occurred at the same rate with or without K^+ in the medium. In contrast, the basal efflux of Rb^+ occurred by a process requiring energy and external K^+, and was inhibited by vanadate. Based on these observations, the model developed for the transcellular transport of K^+ envisages a basolaterally located ATPase which pumps interstitial K^+ into the Sertoli cell cytoplasm. Subsequently, the cytoplasmic K^+ passes across the apical plasma membrane via a K^+ channel to the extracellular milieu [32]. This process of Rb^+/K^+ transcellular transport was stimulated by forskolin, $(Bu)_2cAMP$ [32, 35] and FSH, suggesting a potential role of the cytoskeleton [32] amongst others, in vectorial transport.

Transferrin mediated transcellular transport of Fe^{2+}



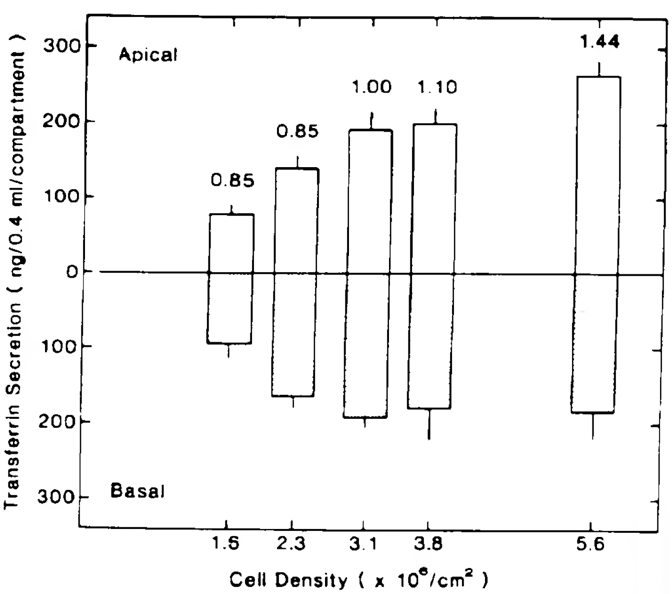


Figure 5. The rate of inulin transport across multichambers in the absence of cells (filter alone) and across confluent epithelial sheets of Sertoli cells at densities of 1.9 - 5.6 x 10^6 cells/cm^2 (A). Sertoli cell density was proportional to electrical resistance and inversely proportional to inulin transport (B).

Figure 6. Sertoli cell density-dependent polarized secretion of transferrin. At each Sertoli cell density is shown the magnitude of transferrin secretion into the apical (open column) and basal (stippled column) reservoirs of the multichambers, and the polarity of transferrin secretion indicated numerically for each cell density. (Figures 5 and 6 modified from Onada *et al.* (1990)[23] and reproduced with permission from the Publisher.)

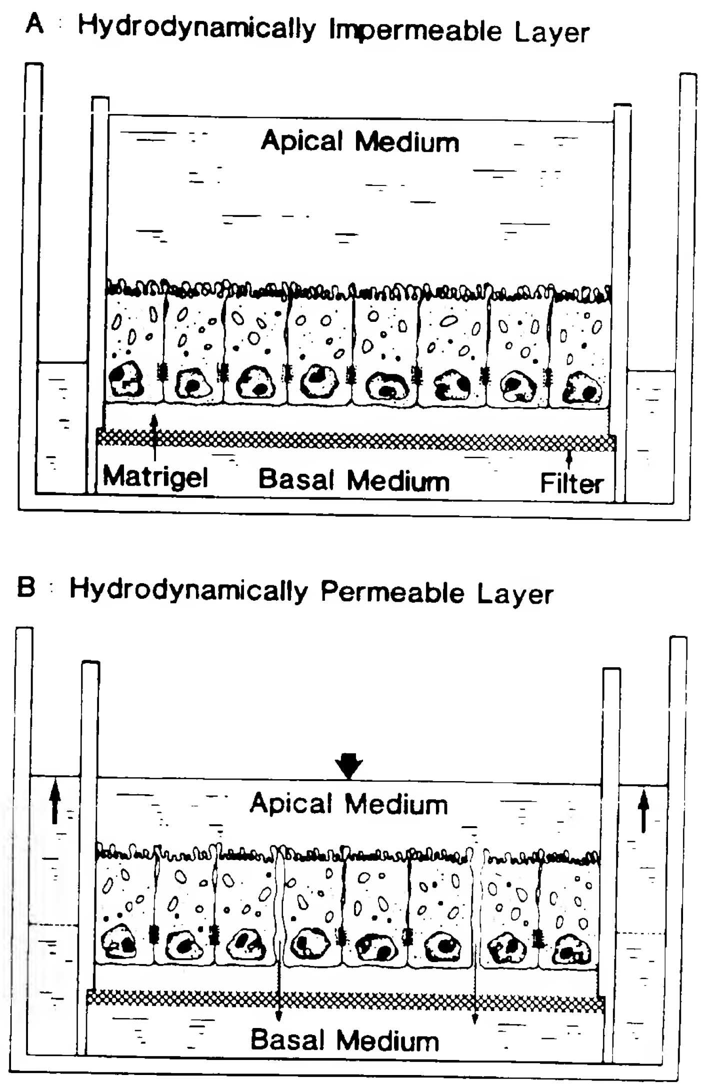


Figure 7. Schematic representation of Sertoli cell epithelial sheets in the multichambers which are able to maintain disparate fluid levels between the apical and basal reservoirs (A, hydrodynamically impermeable) or are hydrodynamically permeable (B) allowing fluid levels to equilibrate. (Modified from Onada *et al.* (1990)[23] and reproduced with permission from the Publisher.)

across confluent epithelial sheets of Sertoli cells grown in multichambers was demonstrated by Djakiew *et al.* [20]. Following addition of human serum ^{59}Fe-transferrin to media bathing the basal cytoplasm of Sertoli cells, rat testicular ^{59}Fe-transferrin was immunoprecipitated from the apically located media overlaying the Sertoli cells. Cross reactivity of the anti-rat transferrin antibody with human transferrin was negligible. This transcellular transport of Fe^{2+} required Sertoli cell viability; the process could be displaced with excess transferrin, and was temperature dependent [20]. In addition, the ^{59}Fe that had been vectorially transported by transferrin across the Sertoli cell epithelial sheets was found to be associated with purified round spermatids and pachytene spermatocytes co-cultured on the apical surface of the Sertoli cells [42]. These results are in accord with the model of Huggenvick *et al.* [9] in which serum holotransferrin is sequestered by a receptor-mediated process into the basal cytoplasm of Sertoli cells, whereupon the Fe^{2+} dissociates from the transferrin carrier protein in the acidified environment of endocytic organelles and is incorporated into *de novo* synthesized Sertoli cell transferrin. The serum apotransferrin recycles out of the basal cytoplasm, whereas the Sertoli cell holotransferrin is secreted from the adluminal compartment of the Sertoli cell and bound by receptors on germ cells or secreted into the lumen of the seminiferous tubule (Fig. 8). This pathway for the transferrin-mediated vectorial transport of Fe^{2+} across Sertoli cells has been examined and confirmed *in vivo* [8] and in organ culture [43, 44]. See also Chapters 7, 8 and 13.

Transport Proteins

The polarized secretion of transferrin in multichambers has been extensively studied by several laboratories. Three independent research groups have reported a predominantly apically directed polarity of transferrin secretion [21, 23, 24, 36, 50]. As shown in Figure 6, the polarity of transferrin secretion increases as a function of plating cell density [23]. At cell densities below 3.1×10^6 cells/cm^2 the polarity of transferrin secretion occurs in a predominantly basal direction, whereas at cell densities above 3.1×10^6 cells/cm^2 the polarity of transferrin secretion occurs in a predominantly apical direction. Indeed, all the studies reporting a predominantly apically directed polarity of transferrin secretion were conducted at cell densities above 3.1×10^6 cells/cm^2 [21, 23, 24, 36, 50]. Conversely, all the studies reporting a predominantly basally directed polarity of transferrin secretion were performed at cell densities below 3.1×10^6 cells/cm^2 [19, 26, 31, 33, 37] and at least some of these cultures were reported to have attained hydrodynamic equilibrium [37]. In any event, there is a consensus that testosterone does not influence the polarity of transferrin secretion [21, 37, 50]. In contrast, FSH, EGF, IGF-I and IGF-II have been shown to reduce vectorial transferrin secretion [50], although, in all cases the polarity of transferrin secretion remained in a predominantly apical direction [50]. Considering the very high concentrations of liver derived transferrin which occurs in the serum (3.7 mg/ml) and testicular lymph (3.7 mg/ml) of the rat [7], it is perplexing to envisage a role for Sertoli cell transferrin secreted predominantly into the interstitium in addition to that of the liver derived transferrin that occurs there in such high concentration. Conversely, considering the isolation of germ cells within the adluminal compartment of the seminiferous epithelium by the Sertoli cell tight junctional complexes, an apically polarized secretion of Sertoli cell transferrin is considered to function in the delivery of Fe^{2+} to the meiotic and post-meiotic germ cells, as has been demonstrated in the multichambers [20, 42], in organ culture of seminiferous tubules [43, 44], and *in vivo* [7,8].

Studies of ABP secretion from Sertoli cells in the multichambers are in general agreement that the polarity of secretion is predominantly in an apical direction [21, 26, 37]. Hadley *et al.* [21] found the apical/basal polarity of ABP secretion to be 4/1 in the presence of testosterone, but this polarity declined to less than 2/1 in the absence of testosterone (Fig. 9). Janecki and Steinberger [26] found the apically-directed polarity of ABP secretion changed

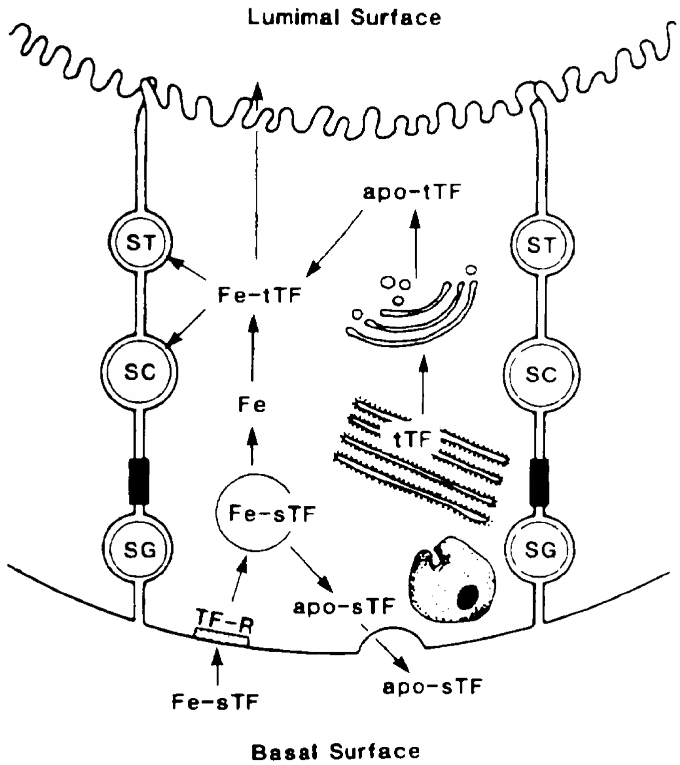


Figure 8. Schematic representation of the transcytosis of iron (Fe) across Sertoli cells in the seminiferous epithelium. Systemic holotransferrin (Fe-sTF) binds to its receptor (TF-R) and is internalized. Upon dissociation of iron the apotransferrin (apo-sTF) recycles to the interstitium. *De novo* synthesised Sertoli cell (testicular) apotransferrin (apo-tTF) incorporates the iron (Fe-tTF) which is transported to adjacent primary spermatocytes (SC) and spermatids (ST) or secreted from the luminal surface of the Sertoli cell.

with the type of culture media, and that this polarity increased with addition of testosterone [37]. Hence, there is a consensus that vectorial ABP secretion occurs in a predominantly apical direction and that testosterone increases the polarity of secretion from Sertoli cells.

Studies of ceruloplasmin secretion from Sertoli cells in the multichambers were carried out by Onoda and Djakiew [39, 40]. A retrospective review of the original immunoprecipitation data indicates that ceruloplasmin is secreted in a predominantly apical direction with an apical/basal secretion ratio of 5.6/1 [39].

Proteases

Polarized secretion of the Sertoli cell protease, plasminogen activator, has been examined using the multichambers [33-35]. Ailenberg and Fritz [33] reported that plasminogen activator secretion occurred in a predominantly basal direction with a polarity of 1/10, and that whereas testosterone did not influence the polarity of plasminogen activator secretion [34], FSH further reduced this polarity to 1/20 [33]. In a subsequent study elevated plasminogen activator levels, elicited by FSH or $(Bu)_2cAMP$, were associated with a decreased integrity of the permeability barrier [35]. This led to the hypothesis that FSH mediates cAMP associated rearrangements of the cytoskeleton which in conjunction with plasminogen activators modify the integrity of the Sertoli cell junctional complexes [35] during remodeling of the seminiferous epithelium.

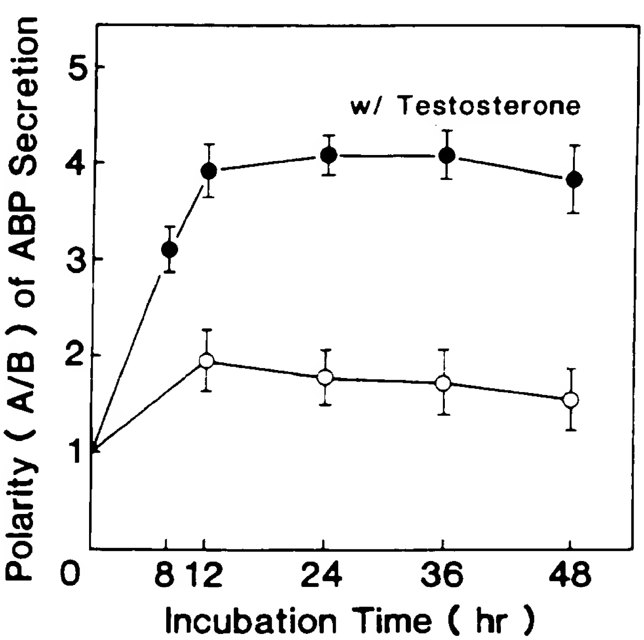


Figure 9. Polarity (apical/basal; A/B) of androgen binding protein (ABP) secretion from Sertoli cells cultured in the multichambers in the presence (closed circles) or absence (open circles) of testosterone over a period of 48 hours. (Modified from Hadley *et al.* (1987)[21] reproduced with permission from the Endocrine Society.)

Endocrine/Paracrine Factors

Polarized secretion by Sertoli cells of the endocrine factor, inhibin, has been studied in the multichambers by several investigators [24, 25, 27, 28, 51]. Handelsman *et al.* [24] reported that rat inhibin is secreted from Sertoli cells with an apical/basal polarity of 4/1, and that this polarity increases to approximately 16/1 upon FSH stimulation. In a comparable study with baboon Sertoli cells, Handelsman *et al.* [25] found the apical/basal polarity of inhibin secretion to be 15/1, which increased to 41/1 with FSH stimulation. They found that inhibin equilibrated across the Matrigel-coated filter in the presence or absence of a Sertoli cell epithelial sheet within approximately 8 hours and could not attribute the predominantly apical polarity of secretion to entrapment and/or binding within the filter [24]. Using similar cellulose culture supports, Janecki *et al.* [27] also found a predominantly apical polarity of inhibin distribution within the multichambers; however, they attributed this result to binding of the inhibin to the Matrigel coated filter support. On polycarbonate filters the polarity of inhibin secretion was reported to be 2/3 predominantly in a basal direction [27, 28, 51], which is similar to the ratio of inhibin secretion calculated by indirect methods for immature rats *in vivo* [12]. In any event, both groups [24, 25, 27] agree that *in vivo* the polarity of inhibin secretion in adult rats is predominantly apically directed, as has been reported from direct [45] and indirect [12] studies.

The only study to examine the polarity of secretion from Sertoli cells in the multichambers of the paracrine factor, which stimulates Leydig cell steroidogenesis, was carried out by Onoda *et al.* [41]. They found this paracrine factor to be secreted from Sertoli cells with a polarity of 1/3 in a predominantly basal direction (Fig. 10). Since leaky chambers could potentially account for a predominantly basal polarity of secretion, these authors were careful to carry out their studies under conditions in which the Sertoli cells had attained and maintained a hydrodynamic permeability barrier, as indicated by the ability of these cultures to maintain higher fluid levels in the apical chamber relative to the basal chamber [Fig. 7]. Indeed, this predominantly basal polarity of secretion of the paracrine factor, which stimulates Leydig cell steroidogenesis (Fig. 10), serves as a control for the predominantly apical secretion of the majority of Sertoli cell proteins that they have investigated [21, 23, 29, 39, 40].

Other Sertoli Cell Proteins

The polarized secretion of SGP-1 from multichambers has been examined by a number of laboratories [29, 36, 40]. Djakiew and Dym [29] examined the polarity of ^{35}S-methionine labeled total protein secretion from Sertoli cells and found that a protein which migrates with similar electrophoretic mobility to SGP-1 occurs predominantly in the apically secreted proteins. In a subsequent study, the identity of SGP-1 was confirmed by immunoprecipitation from the protein secreted apically from Sertoli cells

grown in the multichambers [40]. In a comparable study Ueda *et al.* [36] found that SGP-1, designated S70 by these authors, was secreted with a polarity of approximately 5/2 predominantly in an apical direction. These authors suggested that since they had previously identified SGP-1 associated with spermiogenesis [46], the apical secretion of this protein may represent utilization by the adluminally located spermatids in the seminiferous tubule.

The SGP-2 protein, also known as clusterin, has been shown to be secreted in a predominantly apical direction from Sertoli cells in the multichambers [29, 36, 39, 40]. Ueda *et al.* [36] found that virtually all of the SGP-2 protein, designated S45-S35 by these authors, was secreted from the apical surface of the Sertoli cells. Djakiew and Dym [29] found that whereas most of this protein was secreted in an apical direction, a small proportion of the secreted protein was detected in the basal reservoir.

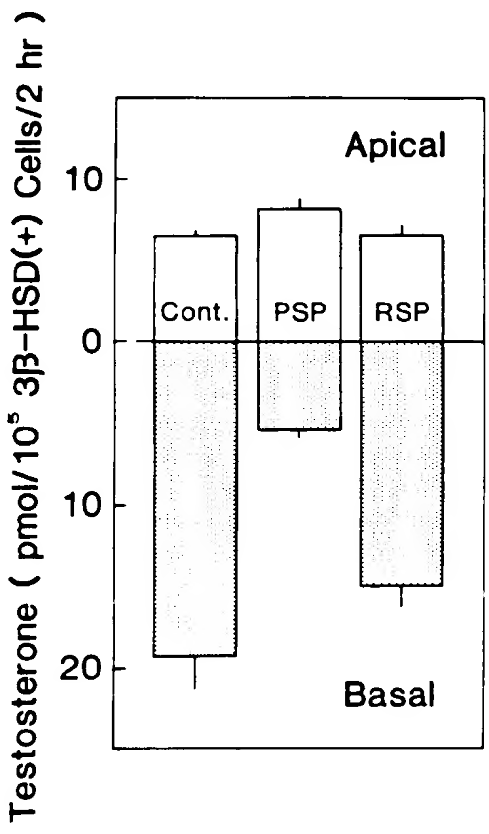


Figure 10. The synthesis and secretion of testosterone from 3-β-hydroxysteroid dehydrogenase (3ß-HSD) positive Leydig cells in response to Sertoli cell conditioned media collected from the apical or basal reservoirs of multichambers in the absence (cont.) or presence of pachytene spermatocyte protein (PSP) and round spermatid protein (RSP) added to the apical reservoir. PSP inhibited the basally directed secretion from Sertoli cells of the factor which stimulates Leydig cell steriodogenesis. (Modified from Hadley *et al.* (1987)[21] reproduced with permission from the Endocrine Society.)

Curiously, the SGP-2, which was secreted from the basal cytoplasm of the Sertoli cells, mostly consisted of the incompletely processed form of the SPG-2 precursor protein [29]; and hence, the basally directed secretion of the precursor protein may have resulted from its missorting in the secretory pathway. Based on a retrospective review of the data of Onoda and Djakiew [39, 40], in which they studied SGP-2 secretion from Sertoli cells in the multichambers, it appears the polarity of secretion of this protein is approximately 1.5, predominantly in an apical direction.

Two other proteins, SP1 [36] and testins [39], are also secreted bidirectionally from Sertoli cells grown in the multichambers. Interestingly, a review of the original data of the polarized secretion of testins [39] and the results of Ueda *et al.* [36] on bidirectional SP1 secretion from Sertoli cells indicate that both of these proteins are secreted in a non-polarized manner with approximately equal amounts of protein occurring in the apical and basal reservoirs of the chambers.

Cell-to-Cell Interactions in Multichamber Systems

Sertoli cells *in vivo* interact with other cell types within the testis by direct cell contacts and through paracrine mechanisms mediated by their secretory proteins. The major cell types with which Sertoli cells interact within the testis include the meiotic and post-meiotic germ cells residing in the adluminal compartment of the seminiferous epithelium, spermatogonia in the basal compartment of the seminiferous epithelium, myoid cells which lie beneath the Sertoli cells, and the Leydig cells which occur in the interstitial space between seminiferous tubules. Some of these interactions that have been examined in the multichambers include the influence of germ cells on Sertoli cells, of germ cells indirectly through Sertoli cells on Leydig cells, and of peritubular myoid cells on Sertoli cells.

Influence of Germ Cells on Vectorial Sertoli Cell Secretions

The influence of round spermatid proteins (RSP) and pachytene spermatocyte proteins (PSP) on the total Sertoli cell protein secreted bidirectionally from the multichambers (Table 1) has been examined by Onoda and Djakiew [39, 40]. They found that the total Sertoli cell secretory protein was stimulated by RSP 133% above control levels, and by PSP 119% above control levels [40]. Interestingly, Sertoli cell secretion was only stimulated by PSP when added to the apical reservoir, but not when added to the basal reservoir [40]. This indicated that the receptor/binding sites for the stimulatory pachytene spermatocyte protein(s) may occur specifically on the apicolateral plasma membrane of the Sertoli cells. Moreover, the influence of these germ cell proteins was additive with FSH in stimulating Sertoli cell protein secretion (Table 1).

Whereas FSH did not influence the polarity of Sertoli cell protein secretions, PSP partially increased the polarity of secretion [40], although not significantly, and RSP more than doubled the polarity of secretion (Table 1). Hence, not only did the germ cell proteins more than double the amount of secreted Sertoli cell protein, but more of the secreted Sertoli cell protein was redirected in an apical direction wherein the germ cells would reside *in vivo*. Within this pool of total Sertoli cell secretory protein, specific proteins such as transferrin [39, 40, 47], SGP-1 [40], SGP-2 [39, 40], ceruloplasmin [39, 40], testins [39] and ABP [47] were secreted in a more apically polarized manner in response to germ cells. Clearly, germ cell modulation of the overall magnitude and polarity of Sertoli cell secretion is consistent with a paracrine interactive modulation of spermatogenesis.

Pachytene spermatocytes, but not round spermatids (Fig. 10), modulate the secretion of the Sertoli cell paracrine protein which stimulates Leydig cell steroidogenesis [41]. This paracrine protein is secreted from Sertoli cells with a polarity of 1/3 predominantly in a basal direction (Fig. 10). However, in the presence of pachytene spermatocyte protein (PSP) the basally directed secretion is inhibited, whereas the apically directed secretion is largely unaffected, the result of which is that the polarity of secretion is inverted to 6/5 slightly in an apical direction (Fig. 10). Hence, the pachytene spermatocytes, indirectly through modulation of the magnitude and polarity of the Sertoli cell paracrine factor, regulate Leydig cell steroidogenesis. The indirect pachytene spermatocyte regulation of steroidogenesis could serve as a mechanism for reinitiation of spermatogenesis following damage to germ cells. In this context, loss of pachytene spermatocytes would relieve the inhibition of the basally secreted paracrine factor, which in turn would stimulate steroidogenesis. Enhanced testosterone production could then reinitiate spermatogenesis either directly through spermatogonial proliferation, and/or indirectly through production of testosterone dependent Sertoli cell proteins which interact with germ cells [48, 49].

Influence of Peritubular Myoid Cells on Vectorial Sertoli Cell Secretions

A number of investigators have studied polarized Sertoli cell protein secretions in peritubular myoid cell-Sertoli cell cocultures [31, 33, 35, 36]. In these cocultures the Sertoli cells and peritubular myoid cells were grown on opposite sides of the support, thereby ensuring that the interaction was of a paracrine nature mediated by diffusible substances, rather than by cell-to-cell contacts. Ueda *et al.* [36] found that in Sertoli cell monoculture the polarity (apical/basal) of secretion of transferrin (3.4/1), SGP-1 (2.5/1) and SP1 (1.1/1) declined in coculture with peritubular myoid cells to a polarity of 2.9/1, 2.4/1 and 0.4/1, respectively. Since the peritubular myoid cells may have endocytosed some of the basally directed Sertoli cell protein, the decline in polarity may be an underestimate. Conversely, the effect of peritubular myoid cell coculture with Sertoli cells was to increase the polarity (apical/basal) of plasminogen activator secretion from 0.12/1 in Sertoli cell monoculture to 0.36/1 when cocultured with peritubular myoid cells [33]. This apparent change in the polarity of secretion needs to be tempered with the possibility that some of the basally directed plasminogen activator may have been endocytosed by the peritubular myoid cells, effectively removing it from the basal reservoir, thus resulting in an apparent increase in the polarity of secretion. Clearly, the use of conditioned media from peritubular myoid cells with the Sertoli cells would circumvent endocytosis of Sertoli cell secretory proteins, although the use of viable Sertoli cells and myoid cells in co-culture does have the advantage of allowing dynamic interactions that may not occur with conditioned media.

Table 1
Effect of FSH and/or RSP on Sertoli Cell Protein Secretion

Culture condition	Total Sertoli cell protein[a] (% of control)	Secretion ratio[a] (apical/basal)
Control	100 ± 14	3.42 ± 0.54
Control + FSH	154 ± 9[b]*	4.04 ± 0.56
Control + RSP	233 ± 20[b]*	8.48 ± 0.97[b]*
Control + FSH + RSP	282 ± 30[b]**	5.76 ± 0.47[b]*

FSH (100 ng/ml) was added to both apical and basal chambers, whereas RSP (0.5 ml at 100 μg/ml) was added to the apical reservoir of the multichambers.
Mean ± S.E. radioactivity (CPM) of control cultures was 140,800 ±18,700 (n=3).
[a]The values represent mean ± S.E. obtained from 3 independent experiments. Each experiment contained 2 cultures per replicate.
[b]Significant difference between control and others. **: $p<0.01$, *: $p<0.05$
(Reproductive with permission from the Publisher [39].)

Conclusions

The culture of Sertoli cells in multichambers enables growth of polarized Sertoli cells *in vitro* that exhibit bidirectional endocytosis, transcellular transport, polarized secretion and cell-to-cell interactions that mimic in many respects the same properties of Sertoli cells *in vivo* (Table 2). The advantage of the multichamber culture system is that the culture conditions are well defined so that particular functions of Sertoli cells and cellular interactions can be examined under highly controlled conditions without the confounding effects of unknown constituents in serum and/or interstitial fluid, and without the confounding effects of the other cell types that may not be under immediate consideration. Polarized secretion is consistent with a more efficient production and release of Sertoli cell proteins from specific cellular domains. This directional secretion within the compartmentalized testis ensures appropriate paracrine interactions between cell types. Some of the many direct interactions between germ cells, Sertoli cells, peritubular myoid cells, and Leydig cells gleaned from *in vivo* studies have now been demonstrated using multichamber culture systems. However, it is also apparent that many of these interactions are indirect and involve more than two cell types. The next challenge will be to concurrently reconstitute within multichambers all the major cellular elements of the testis (Fig. 1D) in order to examine their complex interactions in a manner that may more closely mimic the process of spermatogenesis as occurs *in vivo*.

Table 2
Use of the Multichamber Culture System to Examine the Cell Biology of Sertoli Cells

Function	References
Receptor mediated bidirectional endocytosis	[22]
Polarized secretion	[23, 24, 25, 26, 27, 28, 33, 34, 35, 36, 37, 47, 50, 51]
Transcellular transport	[20, 32, 42]
Cell-to-cell interaction	[29, 31, 39, 40, 41]

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